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CONTENTS OF VOLUME LXI.

No. 1. August, 1924.

BAUMANN, EMIL J. A simple method for preparing large quantities of yeast nucleic acid as a magnesium compound 1
DEAS, J. Yeast growth-promoting vitamin tested for its effects on animals 5
LOEBEL, ROBERT O., BARR, DAVID P., TOLSTOI, EDWARD, and HIMWICH, HAROLD E. Studies of the effect of exercise in diabetes. II. Lactic acid formation in phlorhizin diabetes 9
GREENWALD, ISIDOR. Is there a toxin in the blood of parathyroidectomized dogs? 33
HENDRIX, BYRON M., and MCAMIS, AVA J. Alkalosis produced by injections of hydrazine sulfate in dogs 45
ANDERSON, ARTHUR K., and SCHUTTE, HARRY S. The determination of nitrogen in connection with the wet combustion method for carbon 57
BENEDICT, STANLEY R., and THEIS, RUTH C. A modification of the molybdic method for the determination of inorganic phosphorus in serum 63
THEIS, RUTH C., and BENEDICT, STANLEY R. The determination of phenols in the blood 67
DIENES, L. A note on the gravimetric microchemical technique 73
DIENES, L. Further studies on the determination of calcium, magnesium, and phosphorus in animal substances 77
BLUMENSTOCK, JULIUS, and ICKSTADT, ALBERT. A note on the rôle of the liver in parathyroid tetany 91
ANDERSON, R. J., and NABENHAUER, FRED P. A contribution to the chemistry of grape pigments. II. Concerning the anthocyans in Clinton grapes 97
DENIS, W., and BORGSTROM, P. A study of the effect of temperature on protein intake 109
VICKERY, HUBERT BRADFORD. Some nitrogenous constituents of the juice of the alfalfa plant. II. The basic nitrogen 117
DUNN, L. C. The effect of cod liver oil in various amounts and forms on the growth of young chickens 129
DAKIN, H. D. A note on the presence of valine in zein 137
DAKIN, H. D. The formation of *l*-malic acid as a product of alcoholic fermentation by yeast 139
HAWKINS, JAMES A. The acid-base equilibrium of the blood of normal guinea pigs, rabbits, and rats 147

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Levene, P. A., Taylor, F. A., and Haller, H. L. On lignoceric acid. 157
Schmidt, E. G., Peterson, W. H., and Fred, E. B. The formation of *l*-leucic acid in the acetone-butyl alcohol fermentation. Plate 1. 163
Leiboff, S. L. A simplified method for cholesterol determination in blood. 177
Anderson, William E., and Smith, Arthur H. The effect of acute scurvy on the subsequent nutrition and growth of guinea pigs. 181
Nelson, J. M., and Cohn, David J. Invertase in honey. 193
Daniels, Amy L., and Stearns, Genevieve. The effect of heat treatment of milk feedings on the mineral metabolism of infants. 225
Blatherwick, N. R., Bell, Marion, and Hill, Elsie. Some effects of insulin on the carbohydrate and phosphorus metabolism of normal individuals. 241
Baudisch, Oskar, and Welo, Lars A. On the mechanism of the catalytic action of iron salts. I. 261
Hertzman, A. B., and Bradley, H. C. Studies of autolysis. XI. Relation of the isoelectric point to digestibility. 275
Colwell, A. R. Insulin and phlorhizin glycosuria. 289

No. 2. September, 1924.

Chibnall, Albert Charles. Spinacin, a new protein from spinach leaves. 303
Osborne, Thomas B., Leavenworth, Charles S., and Nolan, Laurence S. A note on Dakin's method as applied to edestin. 309
Leavenworth, Charles S. Note on the basic amino acids yielded by casein. 315
Hastings, A. Baird, Sendroy, Julius, Jr., Murray, Cecil D., and Heidelberger, Michael. Studies of gas and electrolyte equilibria in blood. VII. The effect of carbon monoxide on the acidity of hemoglobin. 317
Gram, H. C. Chlorides of serum, blood, and corpuscles in various pathological conditions. 337
Austin, J. Harold. A note on the estimation of carbon dioxide in serum in the presence of ether by the Van Slyke method. 345
Petrén, Karl. Low nitrogen metabolism with low carbohydrate diet in diabetes. 355
Bodansky, Aaron. A study of milk-coagulating enzyme of *Solanum elaeagnifolium*. 365
Hubbard, Roger S., and Wright, Floyd R. Variations in the rate of excretion of the acetone bodies during the day. 377
Jacobs, Walter A., and Collins, Arnold M. Strophanthin. V. The isomerization and oxidation of isostrophanthidin. 387
Steenbock, H., and Black, A. Fat-soluble vitamins. XVII. The induction of growth-promoting and calcifying properties in a ration by exposure to ultra-violet light. 405
Nash, Thomas P., Jr., and Benedict, Stanley R. On the mechanism of phlorhizin diabetes. Second paper. 423

Levene, P. A., and van der Hoeven, B. J. C. The concentration of vitamin B 429
Levene, P. A., Simms, H. S., and Pfaltz, Mimosa H. The relation of chemical structure to the rate of hydrolysis of peptides. I. On the synthesis, on the physical constants, and on the rates of hydrolysis of methylated peptides 445
Ronzoni, Ethel, Koechig, Irene, and Eaton, Emily P. Ether anesthesia. III. Rôle of lactic acid in the acidosis of ether anesthesia 465
Howe, Paul E. The differential extraction and precipitation of the soluble proteins of muscle, with data on the concentration of proteins in the muscle of the calf, cow, and rabbit 493
Van Slyke, Donald D., and Neill, James M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I 523
Harington, C. R., and Van Slyke, Donald D. On the determination of gases in blood and other solutions by vacuum extraction and manometric measurement. II 575

No. 3. October, 1924.

Shaffer, P. A., and Friedemann, T. E. Antiketogenesis. V. The ketolytic reaction; action of glycol aldehyde and of glyoxal 585
Smith, Lawrence Weld. The experimental feeding of dried breast milk 625
Updegraff, Helen, and Lewis, Howard B. A quantitative study of some organic constituents of the saliva 633
Greenwald, Isidor. Some chemical changes in the blood of dogs after thyroparathyroidectomy 649
Wang, Chi Che, and Felsher, Augusta R. The effect of alcoholic extract of commercial granular glucose on urinary reducing substance 659
Quick, Armand J. A method for the quantitative determination of menthol glycuronic acid in urine 667
Quick, Armand J. The synthesis of menthol glycuronic acid in the rabbit 679
Anderson, R. J. A contribution of the chemistry of grape pigments. III. Concerning the anthocyans in Seibel grapes 685
Hastings, A. Baird, and Sendroy, Julius, Jr. Studies of acidosis. XX. The colorimetric determination of blood pH at body temperature without buffer standards 695
Doisy, Edward A., Ralls, J. O., Allen, Edgar, and Johnston, C. G. The extraction and some properties of an ovarian hormone 711
Mattill, H. A., Carman, J. S., and Clayton, M. M. The nutritive properties of milk. III. The effectiveness of the X substance in preventing sterility in rats on milk rations high in fat 729

Youngburg, Guy E., and Pucher, George W. Studies on pentose metabolism. I. A colorimetric method for the estimation of furfural 741
Rose, William C., and Cox, Gerald J. The relation of arginine and histidine to growth 747
Steenbock, H., Hart, E. B., and Jones, J. H. Fat-soluble vitamins. XVIII. Sunlight in its relation to pork production on certain restricted rations 775
Hirsch, Edwin F. Changes in the hydrogen ion concentration of the blood with coagulation 795
Chen, K. K., Meek, Walter, and Bradley, H. C. Studies of autolysis. XII. Experimental atrophy of muscle tissue 807
Austin, J. H., Cullen, G. E., Gram, H. C., and Robinson, H. W. The blood electrolyte changes in ether acidosis 829
Index to Volume LXI 841

A SIMPLE METHOD FOR PREPARING LARGE QUANTITIES OF YEAST NUCLEIC ACID AS A MAGNESIUM COMPOUND.*

By EMIL J. BAUMANN.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, and the Laboratory Division, Montefiore Hospital, New York.)

(Received for publication, May 23, 1924.)

Attempts made several years ago to prepare a large quantity

CORRECTIONS.

On page 650, Vol. lx, No. 3, July, 1924, 2nd line from the bottom, for *5.82 per cent* read *5.92 per cent.*

In the article by Harrop and Benedict in the April number, 1924, Vol. lix, No. 3, the table at the foot of page 696 expresses the same results as Fig. 1 on page 689.

at this time for only a very mild and brief treatment of the yeast with weak alkali is used.

The process depends upon: (1) The concept that nucleic acid exists in yeast as a nucleoprotein and that nucleoprotein is a type of acid protein which is easily separated into its components, nucleic acid and protein, by alkali, forming the alkali salt of the nucleic acid and meta-protein. (2) The solubility of sodium nucleate in dilute acetic acid. (3) The precipitation of nucleic acid as a magnesium salt by magnesium sulfate and hydrochloric acid.

* Presented before the American Society of Biological Chemists, Dec. 27–29, 1917.

The crude preparations contain no protein, but are contaminated with some substance containing carbon and hydrogen but no phosphorus or nitrogen, for carbon and hydrogen estimations are higher and nitrogen and phosphorus values are lower than those that correspond to Levene's formula for yeast nucleic acid. However, the nitrogen to phosphorus ratio of the crude preparations always agrees excellently with the theoretical. The phosphorus to magnesium ratio indicates that the acid is precipitated as a mixture of the mono- and dimagnesium salts.

Attempts at purification have not yielded very satisfactory analytical results; probably some decomposition like that discovered by Steudel and Peiser occurs in the purification process used.

The yields vary from 0.6 to 1.2 per cent of the weight of the wet yeast, depending largely on the freshness of the yeast.

On acid hydrolysis the product behaves typically, adenine and guanine having been easily isolated and identified by analysis.

Description of the Method.

The fresh brewer's yeast is diluted with 2 parts of water and a concentrated solution of crude sodium hydroxide is added with constant stirring, 50 gm. of sodium hydroxide being used per kilo of yeast. Stir vigorously for 5 to 10 minutes.

Sufficient concentrated, commercial hydrochloric acid is then added to neutralize four-fifths of the sodium hydroxide. The remainder of the alkali is neutralized with 30 per cent acetic acid until the reaction is distinctly acid to blue litmus paper. During the neutralization, trouble is experienced with foaming. This may be obviated by the addition of a few drops of amyl alcohol from time to time. The alkaline solution of yeast is light brown. When the end-point is reached, the color is grayish white. During neutralization considerable heat is liberated. This is to be avoided since some decomposition occurs. The yields are improved by keeping the mixture cool. A block of ice added before neutralization is effective.

The mixture is allowed to settle overnight, the supernatant fluid is then siphoned off and filtered through paper pulp.

If large quantities of nucleic acid not quite so pure are desired, the process can be greatly simplified by using the supernatant

fluid without filtration. The sludge may be filtered on large folded filters and additional nucleic acid obtained from this filtrate.

To the filtrate, which is usually slightly opalescent and may give a very slight protein test or be protein-free, magnesium sulfate, $MgSO_4 \cdot 7H_2O$, to the extent of 4 to 5 per cent is added. An aliquot of 500 cc. of this filtrate is taken and concentrated hydrochloric acid, diluted with an equal volume of water, is added from a burette with stirring, until precipitation is complete. *This must be done carefully*, the solution being allowed to become quiescent from time to time to observe whether any flocking out of nucleic acid occurs. When the amount required for complete precipitation has been accurately determined, a proportionate quantity of concentrated hydrochloric acid is slowly added to the bulk of the filtrate with vigorous stirring. The last 25 to 40 cc. of hydrochloric acid should be added only after being certain that precipitation is not complete. An excess of hydrochloric acid will redissolve the nucleic acid forming a milky solution. If this occurs the solution should at once be made alkaline to red litmus and the precipitation with hydrochloric acid tried again. However, in this event successful results are often not obtained.

The nucleic acid agglutinates, forming large flocks in a few minutes. These settle to the bottom of the vessel; the supernatant fluid is siphoned off and discarded. Transfer the precipitate and remaining fluid to a smaller vessel and add an equal volume of 60 per cent alcohol, stir well, allow to settle, and decant. Repeat, washing with 80 and 95 per cent alcohol. Filter finally upon a hardened filter paper, draining thoroughly with the pump. Transfer the substance to a mortar and grind well with sufficient 95 per cent alcohol to give it a consistency of porridge. Filter on the same paper and drain thoroughly. Transfer to a mortar and grind well with ether; filter again on the same paper and dry quickly on a plate above a radiator (45–50°C.), or in a vacuum desiccator. If drying is not rapidly done the substance will hydrate and become brown. When almost air-dried, grind to a powder and dry above radiator completely.

Some of the analyses on different air-dried unpurified preparations are given in the table below. They represent averages of satisfactory duplicate determinations.

No.	N	P	Mg	N:P	P:Mg
1	11.90	6.77		1.76	
2	12.70	7.48		1.70	
3	12.58	7.58	2.09	1.66	3.63
4	11.28	6.78	1.82	1.69	3.72
Theory for monomagnesium salt.....				1.69	5.09
" " dimagnesium "				1.69	2.56

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YEAST GROWTH-PROMOTING VITAMIN TESTED FOR ITS EFFECTS ON ANIMALS.

By J. DEAS.

(From the Department of Pharmacology, University of Toronto, Toronto, Canada.)

(Received for publication, May 5, 1924.)

The yeast growth-promoting vitamin, first described by Wildiers[1] in 1901, though not described by him as such but as a "bios," was long considered to be the same as vitamin B. Casimir Funk and Dubin[2] have shown that the yeast growth-promoting vitamin, called by them vitamin D, can be separated from that which causes growth of rats.

Professor W. Lash Miller, Department of Chemistry of this University, with his assistants has been studying quantitatively the yeast growth-promoting vitamin which for clearness will be referred to throughout this paper as (Wildier's) bios. As recently reported, Dr. G. H. W. Lucas[3] found an abundant and cheap source of this bios in malt combings which consist largely of the rootlets from the sprouted malt. An infusion from these rootlets contains abundant bios with much less solids than is the case if bios is made from malt. Further attempts to isolate the purified bios resulted in the isolation of the two substances, Bios I and Bios II. The first of these may be precipitated with baryta, is not adsorbed on charcoal, nor is it precipitated by lead acetate save in the presence of an excess of ammonia. Bios I is not removed by shaking with yeast. Bios II is adsorbed by charcoal, removed from solution by shaking with yeast, and soluble in acetone.

[1] Wildiers, E., *La Cellule*, 1901, xviii, 313.

[2] Funk, C., and Dubin, H. E., *J. Biol. Chem.*, 1921, xlviii, 437.

[3] Miller, W. L., *Science*, 1924, lix, 197.

Excess of either of these two substances and especially Bios II is detrimental to the growth of yeast. It seemed, therefore, pertinent to examine the rootlets, the infusion, and each of the two partially purified principles separately and together in a balanced mixture such as is sufficient to promote the growth of yeast. As a control rice polishings were used which were also tested against yeast and shown to contain the necessary "bios." The amount of rice polishings used, 1 gm. daily, was equal in bios to 0.4 gm. of dried yeast, which work in this laboratory and elsewhere has shown contains sufficient vitamin B for normal growth. The quantities of rootlets and of the bios preparations were also such as to correspond with this necessary amount of dried yeast. These controls were made by Dr. G. H. W. Lucas in the Department of Chemistry.

Young rats of a weight of 30 to 40 gm. from Wistar Institute stock, bred in the laboratory, were placed on a vitamin-free diet consisting of casein 18 per cent (heated to 130°C. in a current of air for 24 hours), starch 58 per cent (heated to 170°C. in air for 7 to 10 hours), lard (heated to 120°C. and a current of air passed through it for 24 hours), salt mixture[4] 4 per cent, agar-agar 5 per cent, and cod liver oil of tested strength 5.6 mg. daily.

When their growth had quite or nearly ceased they were given the source of "bios." The rootlets were given as a dried powder, 1 gm. daily, placed on top of their daily food supply, which was readily and completely eaten. In this case only one rat was placed in each cage. The infusion was given in 0.5 cc. doses daily and was administered from a hypodermic syringe and by mouth. The rats did not relish it much but with a little persistence could be made to take it. Bios I, 0.35 cc. doses daily, was given in a similar manner and was readily taken. Bios II was given in a dose of 0.33 cc. daily, but with great difficulty. Bios I and II were given separately in the same doses.

The accompanying curves (Fig. 1), two for each preparation, show the extremes that were obtained in each series, due to variations probably in the resistance of the individual rats. They

[4] Salt mixture: (McCollum No. 185 as modified by Stammers (Stammers, A. D., *Biochem. J.*, 1921, xv, 489)) NaCl 46.25, $MgSO_4$ 71.2, NaH_2PO_4 92.68, K_2HPO_4 254.6, $CaH_4(PO_4)_2 \cdot H_2O$ 144.20, Ca lactate 347.0, Fe citrate 31.52, NaFl 0.55, $MnSO_4$ 2.00, Kl 10.

show quite clearly that the rootlets contain little or no vitamin B nor its infusion. Bios I and Bios II, or both in a combination so balanced as to promote normal growth of yeast abundantly, are also inefficient in promoting the growth of rats. The results

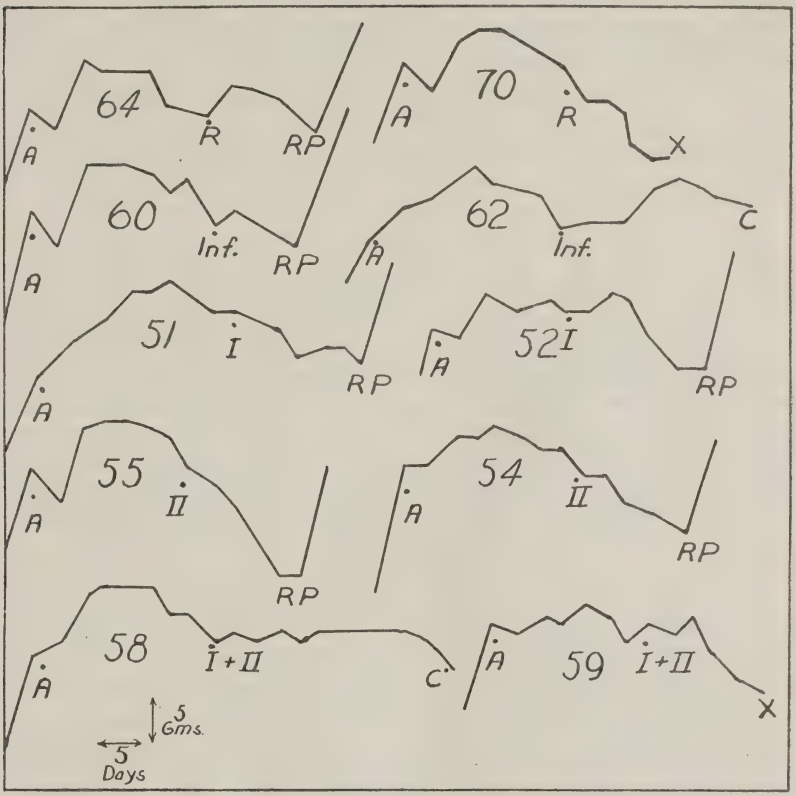

Fig. 1. Dots indicate points of change of diet; A, vitamin-free diet; R, 64 and 70 rootlets; Inf., 60 and 62 infusion of rootlets; I, 51 and 52 Bios I; II, 55 and 54 Bios II; I + II, 58 and 59 combination of bios; R P, rice polishings; X, death; and C, change of diet.

have been referred to in the review published by Professor Miller[3] and also to the fact that these substances do not cure beri-beri in pigeons.

In order to show that neither or both of these bios, had anti-scorbutic value a series of healthy young guinea pigs of about

300 gm. weight were put on a scorbutic diet[5] consisting of ground whole oats 59 per cent, Klim 30 per cent (heated to 110°C. for 5 hours), butter fat 10 per cent, and NaCl 1 per cent.

For the first few days on the scorbutic diet the rats were given carrots as a source of vitamin C, then the basal diet alone, and finally rootlets or Bios I or II, or both Bios I and II. The controls were given orange juice and grew normally and showed no symptoms of scurvy. They were killed and compared with those on bios. Those on rootlets and the bios showed typical symptoms of scurvy and at post-mortem the findings were those of severe scurvy.

Typical Protocol.—Rat 87. Weight 177 gm. Normal diet 20 days, weight at end of period 274 gm. Scorbutic diet and carrots 6 days, weight 296 gm. Scorbutic diet 18 days (44 days), weight 346 gm., not as good growth. Rootlets 1 gm. daily, weight fell, died on 55th day, 29 days on scorbutic diet, weight 210 gm. Symptoms: 50th day moving stiffly, swellings of wrists; 52nd day swellings more marked. Postmortem swellings at costochondral junctions, slight hemorrhages at two costochondral junctions and swellings at costosternal junctions, knees and wrists badly swollen, inflamed and hemorrhagic. Bones brittle, epiphyseal junctions of long bones abnormal. Gums soft and loose and some hemorrhage. Some loose teeth in lower and upper jaws. Adrenals enlarged and pale. Other organs normal.

CONCLUSIONS.

1. It has been shown that malt rootlets and combination infusion of the same and the fractions known as Bios I and Bios II of the yeast growth-promoting vitamin either separately or in combination are insufficient to produce the growth of rats.

2. Consequently Wildier's bios, and Funk's vitamin D, are not identical with the rat growth-promoting vitamin B.

3. It is not identical with the antiscorbutic vitamin C.

The thanks of the author are due to Professor V. E. Henderson, under whose direction the work was carried out, to Professor W. Lash Miller, and Dr. G. H. W. Lucas who kindly supplied the necessary materials for these experiments.

[5] Sherman, H. C., La Mer, V. K., and Campbell, H. L., *J. Am. Chem. Soc.*, 1922, xliv, 165.

STUDIES OF THE EFFECT OF EXERCISE IN DIABETES.

II. LACTIC ACID FORMATION IN PHLORHIZIN DIABETES.

By ROBERT O. LOEBEL, DAVID P. BARR, EDWARD TOLSTOI, and HAROLD E. HIMWICH.

(From the Russell Sage Institute of Pathology in Affiliation with the Second Medical (Cornell) Division and the Department of Pathology of Bellevue Hospital and the Department of Medicine, Cornell University Medical College, New York City.)

(Received for publication, May 5, 1924.)

In a previous publication (1) we have reported the results of observations upon the response of human diabetics to exercise. It was found that as the result of exertion, severe or moderately severe diabetics produce lactic acid which accumulates in the circulating blood. For an equivalent amount of work the accumulation was somewhat greater than that observed in normal individuals. The experiments demonstrated that diabetics of a considerable grade of severity can accomplish work by the normal mechanism of muscular contraction; *i.e.*, by the formation of lactic acid. From the theoretic standpoint, however, the experiments could not be conclusive, since in no case was the diabetes complete. It seemed possible that all the diabetics studied had retained enough of the normal carbohydrate metabolism to accomplish the muscular exercise in the usual manner. It was our desire to determine whether completely diabetic individuals could form lactic acid, and, if not, by what chemical mechanism the process of muscular contraction was accomplished. Complete diabetes, especially since the introduction of insulin therapy, is a great rarity, and in those few complete diabetics who have been carefully observed a partial ability to metabolize carbohydrate may return at any time. Furthermore, on account of the extremely critical condition of such patients exercise sufficient to show lactic acid would not be justifiable. To study the response to exercise during the com-

pletely diabetic state, it, therefore, became necessary to use either phlorhizinized or depancreatized animals.

For this study phlorhizin diabetes was selected, not only because of the greater ease of preparation, but because animals treated in this way are in general stronger and more capable of exercise. Regardless of the mechanism which may be involved in phlorhizin diabetes, it is established that the loss of the function of carbohydrate oxidation is as complete as in any known condition. The non-protein respiratory quotients of completely phlorhizinized animals indicate that fat alone is oxidized and the D:N ratios are the same as in complete human diabetes. They are higher and usually more constant than in animals which have been depancreatized.

In addition to phlorhizin some animals were also given repeated injections of adrenalin on the day preceding the exercise experiment.

Sansum and Woodyatt (2), in extension of the previous work of A.I. Ringer in 1910 give evidence that following adrenalin the glycogen reserves of the body are exhausted even when the period of phlorhizin and fasting corresponds to the shortest that we employed. They found that ether and nitrous oxide caused an increased excretion of glucose in phlorhizin dogs, but not in animals which received adrenalin in addition to phlorhizin. This was interpreted to mean that adrenalin caused a complete exhaustion of the glycogen deposits so that narcotics could no longer induce an increased glycosuria. As another point in favor of this view, they confirmed the findings of A. I. Ringer that in phlorhizinized dogs adrenalin first causes a greater excretion of glucose through the urine with the result that D:N ratios are high. Later doses of adrenalin do not raise the D:N ratios, presumably because there is no more glycogen to be expelled. For our purposes we only insist upon the point that the glycogen store is materially reduced by treatment with adrenalin.

In the following pages an attempt has been made to answer two main questions: (1) May an animal which has lost the power of oxidizing carbohydrate retain the ability to form lactic acid? (2) May lactic acid be formed in a completely phlorhizinized animal whose glycogen stores are minimal? Incidentally, observations have been made on the glucose content of the blood and on the acetone content of blood and urine before and after exercise.

Preparation of Animals.—Since there were no observations in the literature upon the lactic acid response of normal animals to strychnine convulsions, it was necessary to obtain such data.

These animals represent our control group. Animals of another series were fasted and given subcutaneously 1.0 gm. of Merck's phlorhizin in 10 cc. of sterile Mazola oil every 24 hours until the D:N ratios indicated that the diabetes was complete. The phlorhizin had been recrystallized in the laboratory before administration. Two dogs were prepared by a method similar to that described by Sansum and Woodyatt (2). These animals, after 3 days of fasting, were given, subcutaneously, 1.0 gm. of phlorhizin in oil and 15 minims of adrenalin (Parke, Davis and Co.). The adrenalin dosage was repeated every 3 hours for 24, after which the experiment with strychnine was performed. Four dogs were given still more thorough preparation. After 3 days of fasting they received the usual dose of phlorhizin in oil until D:N ratios indicated complete diabetes. Adrenalin, 15 minims, was then administered every 3 hours for 27 hours. From 3 to 6 hours later the usual experiment with strychnine was begun.

Even after prolonged treatment with phlorhizin complete D:N ratios did not appear in five dogs. Of these, two were discarded; the others (Dogs 5, 6, and 9) were used in exercise experiments.

In the individual experiments of each group the details of preparation varied considerably and may be found in the protocols.

Character of Exercise.—It was our original intention to study the effects of work performed on a treadmill. In a short trial, an animal (Dog 7), treated with phlorhizin and also with adrenalin for 1 day, would not continue at work of this sort. Previous experiments both on normal men and normal dogs had shown that a considerable amount of work was necessary before an accumulation of lactic acid could be demonstrated in the circulating blood. Since in our experiments the accumulation was the only criterion of lactic acid formation, it became necessary to use artificial means of causing vigorous muscular contraction. For this purpose, we employed strychnine. Our aim was to produce by carefully graduated dosage a condition of hypersensitivity for a long period before convulsions occurred. In this we were aided by directions given to us by Dr. Robert Hatcher of the Department of Pharmacology. The first dose of strychnine was 0.3 mg. per kilo of body weight; second dose in 15 minutes,

0.1 mg. per kilo; third dose in 15 minutes, 0.05 mg. per kilo; this last to be repeated every 15 minutes until sensitivity was produced. The dosage necessary to produce sensitivity varied greatly in the different animals. After 2.2 mg. one normal dog became hypersensitive. In one of the phlorhizinized animals of almost twice the size 10.3 mg. were necessary before hypersensitivity developed. The degree of sensitivity of the animal was determined by tapping on the table with a percussion hammer. When a stroke of the hammer caused a vigorous contraction of the muscles of all four extremities that time was taken as the beginning of exercise. Work consisted of the response elicited by 60 to 70 taps per minute. When strychnine was given by this method it was possible to maintain a state of hypersensitivity for 1 to 2 hours before death.

Blood Samples.—A sample of blood was always taken before the administration of strychnine. Subsequently, blood was drawn at varying intervals after the exercise had been started: the time of blood taking being regulated as far as possible to correspond to the greatest amount of exercise. In certain experiments several samples were taken to demonstrate different stages in the exertion. The period following a severe tonic convulsion was considered especially desirable. For blood samples the femoral artery and vein were used. In one experiment, not published, a cannula was inserted into the femoral vein. Soon after the vein was tied muscular contractions were reduced to a minimum; apparently the collateral circulation was not sufficient. In the other experiments blood was obtained by needle puncture.

The receivers for blood contained 2.0 mg. of neutral potassium oxalate per 1 cc. of blood, and sometimes, 0.1 mg. of sodium fluoride per cc. The latter was added to check glycolysis. Analyses for sugar and lactic acid were started within 5 minutes of the time the blood was drawn.

Methods.

Lactic acid was determined by the method of Clausen (3). In four of the experiments analysis for blood sugar was made by Benedict's method (4). Later determinations were made

by the method of Folin and Wu (5). The results obtained by this method were uniformly lower. Nitrogen in the urine was done by the Kjeldahl method, sugar in the urine by the method of Benedict (6). Acetone in the urine and blood was determined by the method of Van Slyke (7).

EXPERIMENTAL.

Twelve dogs were studied. They fall into three groups: (1) control, (2) dogs receiving phlorhizin alone, and (3) animals receiving combined phlorhizin and adrenalin treatment. Since we cannot express the exertion of strychnine contractions in terms of mechanical units the degree of exercise will be consistently described as poor, fair, vigorous, and convulsions. In most instances this judgment represents the opinion of the same two observers throughout. In regard to blood samples, it will be understood that unless otherwise specified in the following protocols, arterial blood was withdrawn from the femoral artery and venous blood from the corresponding vein. Also, except where the reverse is explicitly stated the vessels had previously been exposed by incision, so that there could be no doubt whether blood was coming from artery or vein.

Dog 1.—Male. Control. June 14, 1923. Weighed about 6.5 kilos. Poorly nourished. No preparation in the preliminary period. The blood samples were obtained by puncture of the femoral vessels which had not previously been exposed by incision. The sample before exercise probably came from a vein. Analysis: Lactic acid 34.3 mg. Sugar[1] 120 mg. The first dose of strychnine was 2.2 mg. Total 2.6 mg. Hypersensitivity developed after the first dose of strychnine. Exercise was fair. Blood was drawn from the artery in two samples at 25 and 27 minutes after the beginning of exercise. The pooled blood was analyzed. Lactic acid 73.5 mg. Sugar[1] 158 mg. Immediately after arterial puncture the dog developed convulsions and died.

Dog 2.—Male. Control. Jan. 25, 1924. Weighed 9.0 kilos. Received no special preparation. The first blood obtained from the femoral vein showed: Lactic acid 11.9 mg. Sugar 85.4 mg. The first dose of strychnine was 2.2 mg. Total 4.1 mg. Hypersensitivity developed 9 minutes after the first dose. At the same time hyperpnea set in and a respiratory rate of 70 to 80 per minute was maintained until practically the end. During the first 19 minutes exercise was fair and venous blood obtained at the end of

[1] Blood sugar determined by the method of Benedict.

this period gave on analysis: Lactic acid 68.6 mg. Sugar 95.2 mg. For about 37 minutes following, exercise became more vigorous. Then it was poor for the next 27 minutes. In the 83rd minute of exertion, just before the animal died, blood was taken from the femoral artery. This sample was quite dark in color (estimated about 85 per cent saturated with oxygen). Its lactic acid content was 104.3 mg. Sugar 88.0 mg. After death rigor was evident in 25 minutes.

Dog 3.—Male. Control. Jan. 28, 1924. Weighed 6.5 kilos. Received no special preliminary treatment. The first blood was obtained from the femoral vein. Analysis: Lactic acid 41.3 mg. Sugar 118 mg. The initial dose of strychnine was 1.7 mg. Total 10.9 mg. A second sample of venous blood was taken after 53 minutes, when the animal had done an amount of work roughly equivalent to one of the less satisfactory phlorhizin-adrenalin dogs. Analysis: Lactic acid 51.8 mg. Sugar 108 mg. Subsequently, exercise was vigorous. Late in the experiment successively large injections of strychnine were given and 9 minutes before the end the animal went into generalized convulsions. These subsided for a minute during which a third sample of venous blood was drawn 125 minutes after exercise had begun. Analysis: Lactic acid 147.7 mg. Sugar 165 mg. Death followed in 7 minutes. Before 21 minutes had elapsed postmortem rigor was present.

As a rough control of the element of asphyxia a sample of arterial blood was drawn at the time of the second and third venipuncture, and a third sample in the period between. All were bright red.

Dog 4.—Female. Weighed 9.3 kilos. Fasted 8½ days. Phlorhizin for 6 days. The D:N ratios were 3.65 and 3.37 on the 4th and 5th days of phlorhizin. The work experiment was performed on the 6th day of phlorhizin, Jan. 21, 1924. The first blood was obtained from the femoral vein. Analysis: Lactic acid 35.7 mg. Sugar 48.8 mg. The initial dose of strychnine was about 2.2 mg. Total about 5.2 mg. For 12 minutes after hypersensitivity appeared, exercise was vigorous. Arterial blood was taken at the end of that time. Analysis: Lactic acid 40.6 mg. Sugar 108 mg. Subsequently, exercise was only fair. Arterial blood which was drawn in the 81st minute was quite dark. Analysis: Lactic acid 55.3 mg. Sugar 117 mg. Venous blood drawn 2 minutes later was analyzed. Lactic acid 68.6 mg. Sugar 115 mg. The urinary D:N ratios were: Before exercise, 3.48; after, 4.33. Total urinary acetone bodies were 13.6 mg. in 228 minutes before and 52 mg. in the 140 minutes which included the work period.

The rate of glycolysis was determined in the arterial blood sample drawn after 81 minutes of work. In 18 hours at 37.5°C. the blood sugar had dropped from 118 mg. to zero; while the lactic acid had risen from 55.3 to 140 mg.

Dog 5.—Female. Weighed 10.2 kilos. Fasted 7½ days. 8 doses of phlorhizin. D:N ratios: 4th day of phlorhizin, 3.00; 5th, 3.04; 6th (incomplete specimen), 3.03; 7th (incomplete specimen), 2.76. The exercise experiment was performed on the 8th day of phlorhizin, Feb. 6, 1924. Before strychnine, the venous blood sample gave on analysis: Lactic acid 27.3 mg. Sugar 43.5 mg.

First dose of strychnine 2.2 mg. Total 5.7 mg. After 16 minutes of vigorous exertion the animal had convulsions of only moderate intensity. Analysis of venous blood drawn 1 minute later: Lactic acid 75.6 mg. Sugar 62.5 mg. Arterial blood drawn at the same time seemed to have a slight oxygen unsaturation. The animal lived 22 minutes longer, but exercise was poor. Rigor was present 18 minutes after death.

Before exercise the urinary D:N ratio was 4.06; after exercise, 3.03.

Dog 6.—Female. Weighed 6.1 kilos. Fasted 5½ days. Given phlorhizin six times in that period. D:N ratios were: 4th day, 3.14; 5th day, 2.73. The work experiment was performed on the 6th day of phlorhizin, Feb. 11, 1924. Analysis of arterial blood before: Lactic acid 42.0 mg. Sugar 47.6 mg.

Initial dose of strychnine 1.9 mg. Total 4.1 mg. The muscular contractions were vigorous and after 19 minutes the dog seemed ready to go into a convulsion. Arterial blood was then drawn. Analysis: Lactic acid 68.6 mg. Sugar 69 mg.

O_2 saturation was 85.6 per cent (determined by Van Slyke-Stadie technique for O_2 capacity and content (8)). Exercise became fair and in the 51st minute a third arterial blood was drawn. It was bright red. Analysis: Lactic acid 114.1 mg. Sugar 123 mg.

The animal died 28 minutes later. Postmortem rigor appeared in 15 minutes. The urinary D:N ratio was 3.08 before exercise; and 3.35 after; in a specimen which represented little more than bladder washings.

Dog 7.—Male. Weighed 10.0 kilos. Fasted 1½ days. 2 doses of phlorhizin, 7 doses of adrenalin, and 1 of suprarenalin (Metz) were given during that time. The animal reacted with shivering, nausea, and vomiting. Then the exercise experiment was performed (June 6, 1924). Blood was obtained by puncture without previous exposure of the femoral artery. Analysis: Lactic acid 15.4 mg. Sugar[1] 83.3 mg. Total acetone bodies 25.0 mg. The initial dose of strychnine was 3.3 mg. Total 16.7 mg. No hypersensitivity was present until 10.3 mg. had been given. Exercise was very satisfactory. After about 10 minutes of work arterial blood was drawn. Analysis: Lactic acid 60.2 mg. Sugar[1] 88.2 mg. Total ketone 20.0 mg. Exercise continued vigorous. About 20 minutes from the start the dog had a tonic spasm and breathing ceased. Blood was drawn from the heart, which was still beating. Analysis: Lactic acid 56.0 mg. For a considerable portion of the experimental period this dog had shown marked hyperpnea.

Dog 8.—Female. Weighed 7.5 kilos. Fasted 2 days. 9 doses of adrenalin given in 27 hours. Phlorhizin given twice in the same period. Urinary D:N ratios in consecutive short period specimens were: 4.96, 3.04, 2.73, 2.55, and 2.42. The exercise experiment was performed June 12, 1924. Analysis of arterial blood before: Lactic acid 25.2 mg. Sugar[1] 90 mg. Total acetone bodies 9.8 mg.

The initial dose of strychnine was 2.2 mg. Total 8.9 mg. After 20 minutes of fair exercise another arterial sample was obtained. Analysis: Lactic acid 29.4 mg. Sugar[1] 94 mg. Total ketone 1.6 mg.

Before exercise the urinary D:N ratio was 2.32; after exercise 2.41. The

total ketone excretion in 78 minutes preceding exercise was 10.2 mg., and in 115 minutes including the work period it was 40.0 mg.

Dog 9.—Female. Weighed 14 kilos. Fasted 9 days. Phlorhizin nine times. Stole food on 5th and 6th days. The urinary D:N ratio for the 12 hour night period of the 7th day was 3.4. The following 24 hour specimen gave a D:N ratio of 3.02. Following adrenalin every 3 hours the ratios of consecutive short period samples became 3.76, 6.95, 9.85, 10.1, 7.04, 5.02, 3.41, 3.11, and 3.28. The work experiment was done on the 9th day of phlorhizin, June 22, 1923. Before exercise an arterial sample was obtained by puncture without previous exposure of the vessels. Analysis: Lactic acid 30.8 mg. Sugar[1] 75.0 mg. Total ketone 21.3 mg. After 24 minutes of vigorous work a second arterial sample was drawn. Analysis: Lactic acid 32.9 mg. Sugar[1] 83.3 mg. Total ketone 17.0 mg. Soon after, large successive doses of strychnine were given intramuscularly. In the 39th minute of work the dog went into a tonic spasm; respiration began to fail and a highly unsaturated sample of blood was obtained. It may have come from artery or vein. Analysis: Lactic acid 77.7 mg.

The urinary D:N ratio before work was 3.52; after, 3.72. The total acetone excretion for 125 minutes preceding strychnine was 42.0 mg., in 85 minutes including the work period it was 11.0 mg.

Dog 10.—Female. Weighed 7.8 kilos. Fasted 7½ days. Received five daily injections of phlorhizin. On the last day received adrenalin subcutaneously for 9 doses. The urinary D:N ratios were: 3rd day, 2.92; 4th day, 3.85. Short period specimens during adrenalinization gave consecutively: 4.41, 2.59, and 2.40. The exercise experiment was performed on the 5th day of phlorhizin, Jan. 9, 1924. An arterial blood was obtained before. Analysis: Lactic acid 48.3 mg. Sugar 67.1 mg. The initial dose of strychnine was 2.2 mg. Total 5.2 mg. After 73 minutes of poor exercise venous blood was drawn. Analysis: Lactic acid 66.5 mg. Sugar 66.6 mg. Exercise became worse as the experiment proceeded. About 10 minutes before the last blood was withdrawn the fore and hind limbs became perfectly flaccid. The last blood was obtained from the artery 92 minutes after work had begun. It was bright red. Analysis: Lactic acid 88.2 mg. Sugar 53.0 mg. Rigor had not developed in the hind legs 20 to 30 minutes after death.

The urinary D:N ratio before was 3.69; after, 3.73. The total acetone bodies excreted during 132 minutes before exercise was 153 mg.; in the next 141 minutes which included the work period, 58 mg. were excreted.

Dog 11.—Female. Weighed 8.6 kilos. Fasted 8½ days. Received phlorhizin for 6 days. The urinary D:N ratios were: 4th day, 3.14; 5th day, 3.23. On the next day adrenalin was given every 3 hours and the D:N ratios on two specimens were 3.18 and 2.90. The exercise experiment was performed on the 6th day of phlorhizin, Jan. 16, 1924. The blood was venous. Analysis: Lactic acid 28.4 mg. Sugar 41.6 mg. The initial dose of strychnine 2.2 mg. Total 5.6 mg. For 17 minutes exercise was poor. Analysis of arterial blood drawn at that time: Lactic acid 28.0 mg. Sugar 48.7 mg. Then, by direct stimulation of all four extremities and by opposing the

contractions with the hands a fair amount of work was performed. Venous blood was drawn 46 minutes after work had begun. Analysis: Lactic acid 29.4 mg. Sugar 42.5 mg.

Exercise lapsed; became poor. A final arterial blood was obtained 83 minutes from the start. Analysis: Lactic acid 28.0 mg. Sugar 43.4 mg.

The urinary D:N ratios were: Before, 2.74; after, 3.16. Total urinary acetone bodies during 186 minutes which included the work period were 221 mg.

Dog 12.—Female. Weighed 10 kilos. Fasted 8 days. Given six injections of phlorhizin. On the last day adrenalin was injected intramuscularly every 3 hours. The reaction was not noticeably more severe than that following subcutaneous administration. The urinary D:N ratios

TABLE I.

Control Series.

Dog No.	Duration of exercise.	Source of blood.	Lactic acid.	Sugar.	Quality of exercise.
			mg. per 100 cc.	*mg. per 100 cc.*	
1	Before.	Vein (?).	34.3	120*	Fair. 2nd blood taken immediately before a convulsion.
	25 and 27 min.	Artery.	73.5	158*	
2	Before.	Vein.	11.9	85.4	Fair for 19 min. Vigorous to 57th min. Then poor to the 83rd min.
	19 min.	"	68.6	95.2	
	83 "	Artery.	104.3	88.0	
3	Before.	Vein.	41.3	118	Poor for 53 min. Then vigorous to 124th min. Generalized convulsion 124th min.
	53 min.	"	51.8	108	
	125 "	"	147.7	165	

*Blood sugar determined by the Benedict method. Folin-Wu method employed in other experiments.

were: 3rd day, 3.64; 4th day, 3.80. Following adrenalin the ratios were: 7.11, 5.65, 3.79, 2.07, and 3.05. The exercise experiment was performed on the 6th day of phlorhizin, Dec. 7, 1923. Blood before was venous. Analysis: Lactic acid 55.6 mg. Sugar 51.2 mg. Exercise was poor for about 75 minutes after hypersensitivity appeared and then failed completely. Arterial blood was drawn about 77 minutes after work. Analysis: Lactic acid 53.5 mg. Sugar 62.5 mg.

The urinary D:N ratio before exercise was 3.07; after, 3.53.

In Table I will be found a summary of the lactic acid and glucose content of the blood, before and after exertion in control

animals. In these normal dogs fair or vigorous muscular contractions were obtained in each case. Convulsions preceded death in two animals. Blood lactic acid mounted progressively during the course of the exercise. The smallest total increase was 39.2 mg. The greatest was 106.4 mg. The sugar content of the blood increased markedly in two experiments; and to a slight extent in the third. In Dog 2 hyperpnea was extreme, and, in addition, the final blood sample showed obvious oxygen unsaturation.

Table II gives a summary of the results on phlorhizinized dogs. Two of these received phlorhizin for 6 days, and one for

TABLE II.

Phlorhizin Series.

Dog No.	Duration of exercise.	Source of blood.	Lactic acid.	Sugar.	Quality of exercise.
			mg. per 100 cc.	*mg. per 100 cc.*	
4	Before.	Vein.	35.7	48.8	Vigorous for 12 min. Fair for the rest of the period.
	12 min.	Artery.	40.6	108	
	81 "	"	55.3	117	
	83 "	Vein.	68.6	115	
5	Before.	"	27.3	43.5	Vigorous. Mild convulsion beginning in 16th min.
	17 min.	"	75.6	62.5	
6	Before.	Artery.	42.0	47.6	Vigorous. Ready to go into a convulsion at 19th min. Fair to 51st min.
	19 min.	"	68.6	69	
	51 "	"	114.1	123	

8 days. In Dog 4 the D:N ratios before exercise were very close to the theoretic. The D:N ratios in Dogs 5 and 6 are somewhat low. In the absence of ideal D:N ratios it may be pointed out that the experiments of Lusk (9) show that after 2 days of phlorhizin the non-protein respiratory quotients indicate no carbohydrate combustion. Dogs with D:N ratios as much above the ideal as ours are below yield respiratory quotients indicative of complete diabetes. Finally, Dogs 4, 10, and 11 gave almost perfect D:N ratios after treatment with the same lot of phlorhizin.

In this group of experiments muscular contractions were

scarcely less vigorous than in the normal. Blood lactic acid rose progressively during exercise. The smallest total increase was 32.9 mg. The greatest was 72.1 mg. The resting blood sugar was low and during exercise showed an increase in each

TABLE III.

Phlorhizin-Adrenalin Series.

Dog No.	Duration of exercise.	Source of blood.	Lactic acid.	Sugar.	Quality of exercise.
			mg. per 100 cc.	*mg. per 100 cc.*	
7	Before.	Artery.	15.4	83.3*	Exercise vigorous throughout. Tonic convulsion beginning about 20th min.
	About 10 min.	"	60.2	88.2*	
	About 20 min.	Right heart.	56.0		
8	Before.	Artery.	25.2	90*	Fair.
	20 min.	"	29.4	94*	
9	Before.	"	30.8	75.0*	Vigorous throughout. Tonic convulsion about 39th min.
	24 min.	"	32.9	83.3*	
	41 "	" (?)	77.7		
10	Before.	"	48.3	67.1	Poor throughout. Practically no exercise in last 5 min.
	73 min.	Vein.	66.5	66.6	
	92 "	Artery.	88.2	53.0	
11	Before.	Vein.	28.4	41.6	Poor for 17 min. Direct stimulation of extremities was fair to 46th min. Poor to 83rd min. No stimulation from 83rd to 95th min.
	17 min.	Artery.	28.0	48.7	
	46 "	Vein.	29.4	42.5	
	95 "	Artery.	28.0	43.4	
12	Before.	Vein.	55.6	51.2	Poor. None at all during the last 2 min.
	About 75 min.	Artery.	53.5	62.5	

*Blood sugar determined by the Benedict method. Folin-Wu method employed in other experiments.

successive blood sample. The difference between the first and final blood sugar value was even greater than in the normal. As to asphyxia, it may have been a factor in the third and fourth

blood samples taken from Dog 4. Also, the rate of glycolysis in this dog deserves mention. In 18 hours at 37.5°C. sugar was completely gone and lactic acid had mounted in proportion.

Table III summarizes the results obtained after the administration of adrenalin to phlorhizinized dogs. The period of fasting and phlorhizin administration varied greatly in different animals. Adrenalin was given in nine doses at 3 hour intervals; the last dose being given 3 to 6 hours before the commencement of the work experiment. The general condition of all the animals after receiving adrenalin was very poor.

Of the two dogs whose combined phlorhizin and adrenalin treatment lasted less than 36 hours, one—Dog 7—exercised vigorously and increased the blood lactic acid by 40.6 mg. during work. The second—Dog 8—did a fair amount of work, but showed no appreciable increase in lactic acid.

Of those animals which received adrenalin after long preparation with phlorhizin, only one—Dog 9—was able to perform vigorous work. Lactic acid increased 46.9 mg. over the initial value. This animal had stolen food 3 days before the work experiment; but adrenalin had only been started after two successive D:N ratios had shown that no excess sugar was being excreted. At most, the food might have added to her glycogen store. It could not have improved her power to burn glucose.

Dog 11 did a fair amount of work without any lactic acid accumulation in three samples drawn at various times during exercise. The other two long term phlorhizin-adrenalin dogs did little work, and showed no significant change in lactic acid.

In all six cases of this series the resting blood sugar was low. Exercise caused no constant or significant change. Marked oxygen unsaturation was noted in the last blood sample in Dogs 7 and 9. In Dogs 7, 8, and 9 the total blood ketones were determined. They fell somewhat during exercise, but the number of observations is too few to have much weight. The low D:N ratios after repeated injections of adrenalin in phlorhizinized dogs have been noted by Sansum and Woodyatt.

DISCUSSION.

The outstanding fact in the preceding experiments is that lactic acid may be formed in an animal which is unable to oxidize

carbohydrate. It also appears in a completely phlorhizinized animal whose glycogen stores have been reduced to a minimum. In the three dogs which were fasted and made diabetic with phlorhizin the results are in agreement, and, in each observation, there is undoubted evidence of lactic acid formation. The results in the group where adrenalin treatment was added, are not so uniform. It is clearly demonstrated in Experiments 7, 9, and 10 that after such treatment lactic acid may be formed. In the other three, no accumulation of lactic acid was noted. These latter results do not rule out the possibility of lactic acid formation, since accumulation in the blood depends upon two factors. First, sufficient must be produced to accumulate in the muscle and, then escape into the circulation; and second, the rate of diffusion of lactic acid into the blood must be greater than the rate of removal from the blood. In two of the dogs on which negative results were obtained there was not enough exercise. Contractions were feeble and no lactic acid accumulation was expected. In Dog 9 the muscular contractions were also weak, but it was possible during one period to elicit, by direct stimulation, an amount of work which was deemed sufficient to cause accumulation. Three blood samples taken at various stages showed no deviation from the resting level. This result is puzzling and is the only one which might be fairly interpreted as a possible inability on the part of the animal to form lactic acid.

The significance of lactic acid formation in these experiments is not entirely clear but has sufficient bearing on several important questions to justify discussion.

Relation of Lactic Acid to the Combustion of Carbohydrate.

Lactic acid appears in many biological processes. It is formed during muscular contraction in normal animals. It has been found to accumulate in several pathological conditions (10) and following the administration of many drugs (11). It is formed in perfusing the livers of normal animals. In shed blood it appears during glycolysis, and in muscle, during post-mortem rigor. The suggestion has been made that it is a normal intermediary in the oxidation of carbohydrate, and as an extension of this hypothesis, it has been suggested that in phlorhizin diabetes the organism cannot oxidize carbohydrate because it cannot

form lactic acid (12). Four of the six observations of Embden and Isaac (13) with liver perfusion in phlorhizinized animals indicated that the excised liver had lost its normal ability to change glucose to lactic acid. Lépine and Boulud (14) found that glycolysis was diminished in phlorhizin diabetes. However, our single glycolysis experiment (Dog 4) gives no indication of an invariable difficulty in the formation of lactic acid from glucose in the test-tube. There is, moreover, in the literature, a considerable amount of evidence, which seems to support the validity of our results. Milne and Peters (15) found no diminution of glycolysis in the blood of one phlorhizinized, and many depancreatized dogs. Embden, Schmitz, and Meincke (16) demonstrated that after 4 days of phlorhizin lactic acid was present in dog's muscle. On standing, as much lactic acid was formed as by the muscle of normal, well fed animals. Even after strychnine convulsions in phlorhizinized dogs one-half of the normal amount of lactic acid appeared.

These results indicate that in phlorhizin diabetes lactic acid may be formed from carbohydrate. The experiments of Mandel and Lusk (17) and of Embden, Schmitz, and Meincke (16) show that the reverse process, glucose formation from lactic acid, may take place. In phlorhizin diabetes, therefore, the defect is not in the reaction glucose $\rightleftarrows$ lactic acid. The question of the actual site of the defect cannot be answered by these experiments.

There is a further question whether lactic acid is under any circumstances an intermediary in the oxidation of carbohydrate, and the evidence upon this point is scanty. There are *in vitro* experiments (18), which show that lactic acid is formed during the decomposition of glucose in strongly alkaline solution. Subsequent addition of hydrogen peroxide leads to the combustion of the other products, but not of the lactic acid. If hydrogen peroxide and alkali are added together to a glucose solution, oxidation occurs without the appearance of lactic acid. Even if such results be applicable to the problem of *in vivo* oxidation, they lend no support to the view that lactic acid is an intermediary.

By injecting large doses of insulin in normal dogs, Briggs, Koechig, Doisy, and Weber (19) obtained a great lowering of the blood sugar and an increase in lactic acid. These results have

been interpreted to indicate that the defect in diabetes lies in the formation of lactic acid from glucose. More recently, Toenniessen (20) reported that the addition of insulin causes an increased formation of acetaldehyde in blood, and also, in the combined pulps of muscle and pancreas to which lactic acid has been added. It has not been demonstrated that the acetaldehyde increased at the expense of lactic acid. The experiments of Noble and Macleod (21) may have some bearing on this question. They found that injection of racemic sodium lactate did not relieve the symptoms of insulin shock in two dogs. If lactic acid is an intermediary in the normal combustion of carbohydrate it is not easy to explain why it fails to exert as beneficial an action as glucose. This evidence makes it seem not unlikely that the oxidative and anoxidative breakdown of carbohydrate may proceed along separate paths. If that should prove to be the case, the defect in phlorhizin diabetes would lie in the oxidative breakdown. The anoxidative breakdown of carbohydrate to the lactic acid would not be involved.

Chemical Mechanism of Muscular Contraction.

The question arises whether the accumulation of lactic acid, observed by us, was associated with the process of muscular contraction. A very strong argument in favor of this idea is that the increase seems to coincide with the violence and duration of exercise. Several other factors which might conceivably lead to lactic acid formation should not be dismissed without discussion. Such factors are: asphyxia, hyperpnea, strychnine, and, in some experiments, adrenalin. It may have been noted that in two experiments the dog had stopped breathing at the time that the lactic acid accumulation was noted. Macleod (22) has noted that during rest the prolonged asphyxiation of etherized dogs causes an increase of lactic acid in the blood. In our animals the period of asphyxia did not last more than 1 to 3 minutes. Two unpublished observations on intense asphyxia of 3 minutes duration in a resting phlorhizinized dog yielded no increase in lactic acid. During exercise, however, a brief period of asphyxia might cause an abnormally great accumulation in the blood, for Fletcher and Hopkins (23) have shown that the

absence of oxygen during contraction causes a lactic acid accumulation in the muscle. In this case, however, the contractile process causes the production of lactic acid and asphyxia merely prevents its removal. Therefore, in two experiments asphyxia may have been a factor in the accumulation. In the other observations it was absent.

The factor of hyperpnea may next be considered. In a single normal animal—Dog 2—great hyperpnea was noted. In many of the animals the breathing was irregular, and, occasionally, there were short periods of overventilation. Since none of our phlorhizinized dogs reacted to strychnine with overbreathing it seems unnecessary to dwell on this factor.

In these experiments muscular contractions were obtained by producing a state of strychnine hypersensitivity. In view of the number of drugs which cause an increased excretion of lactic acid in the urine it might be suggested that strychnine itself causes a lactic acid acidosis independent of muscular contraction.

There are two reasons for not accepting this explanation for the total increase in lactic acid. While in the normal dogs it is conceivable that an obscure action of strychnine contributed to the accumulation of lactic acid, the exercise was violent and must have caused an overflow of lactic acid into the blood. The final figures were of the order that one expects with such grades of exertion under physiologic conditions. The accumulation of lactic acid was scarcely more than was found in the phlorhizinized animals and corresponded to the somewhat greater degree of exertion. The results give no indication that there was in the normal animals a summation of a "direct" strychnine effect and of the effect of muscular exertion. In the second place, there is little warrant for assuming that a phlorhizinized animal would lose the power to form lactic acid from glycogen in the process of muscular contraction, while preserving the ability to produce lactic acid in some other possible response to strychnine.

Unpublished experiments of Loebel and Tolstoi show that 1 hour after the injection of adrenalin, there is in man a moderate lactic acid accumulation in the blood. Where adrenalin was given in the experiments reported in the present paper the exercise did not follow until 3 or 6 hours after the last dose. Adrenalin

may have affected the resting values, but it is not likely that it would have caused the increases which occurred following the strychnine administration.

There seems little reason to doubt that the lactic acid accumulation which is seen in these experiments arose from the contraction of muscle. This is important for it indicates that the usual lactic acid mechanism is responsible for muscular contraction in completely phlorhizinized animals, and even in those which are completely phlorhizinized and have minimal glycogen stores.

Source of the Lactic Acid.

From consideration of chemical structure and from a mass of *in vitro* and *in vivo* evidence it is well established that under ordinary conditions lactic acid may arise from carbohydrate or from the carbohydrate fraction of the protein molecule. There is little evidence to indicate that it arises from the breakdown of fat. We purpose to consider whether in our animals the well established sources of carbohydrate could supply the lactic acid, or whether it is necessary to assume that any of the lactic acid proceeded from fat.

In the dogs treated with phlorhizin alone it seems entirely possible that the lactic acid may have arisen from a store of glycogen (24, 25).

The experiments of Sansum and Woodyatt make it seem unlikely that glycogen could have been the source of lactic acid in those dogs to which adrenalin was administered. Even after adrenalinization, however, it is possible that a carbohydrate precursor of lactic acid may still be present in the muscle, for Embden and Isaac (13) have shown that in dogs, despite prolonged fasting and treatment with phlorhizin and strychnine, the muscles retain a considerable store of substance—other than glycogen—which will produce lactic acid. Furthermore, in our experiments after the most thorough treatment, the blood sugar never fell below a level of about 40 mg. per 100 cc. A certain level of blood sugar is maintained. From analogy it does not seem unlikely that in the muscles a minimal store of lactic acid precursor is maintained to the last moments of life.

Another possibility is that even though the muscle's original

store of carbohydrate was completely exhausted because of adrenalin administration, a new supply might be elaborated from protein. This might be utilized to form a new store of glycogen before the experiment, or the glucose derived from protein broken down during the experiment might go through a lactic acid stage. There is some indirect evidence which possibly favors the view that during rest a portion of the glucose from protein is stored. As noted by Sansum and Woodyatt, and by us, the D:N ratios in certain experiments assume a definitely lower level after the administration of adrenalin. This may be explained by assuming that part of the carbohydrate fraction of the protein molecule is retained to form a new glycogen store.

It seems sufficiently clear that as long as an animal lives there is a possible carbohydrate source of lactic acid in the body, and it is, therefore, by no means necessary to assume that the lactic acid proceeds from any other source.

The possibility that carbohydrate or lactic acid may be formed from fat cannot be neglected. It is a well known fact that fat may be formed from carbohydrate and *a priori* it might seem equally easy for the body to accomplish the opposite transformation of fat into lactic acid or lactic acid precursor. For this, however, no direct evidence has been adduced. The exact and important experiments of Krogh and Lindhard (26) are interpreted as indirect evidence that such a transformation does occur. With a high fat, low carbohydrate diet, work was accomplished less efficiently than with a low fat, high carbohydrate diet. The diminished efficiency and certain changes[2] in the respiratory quotient during exertion led them to advance the hypothesis that fat was converted into carbohydrate when the carbohydrate in the food was limited. The chief obstacle to the acceptance of this idea has been the work of Stiles and Lusk (27) who found that D:N ratios remain quite constant in phlorhizinized animals during rest and exercise. This constancy was taken as an indication that the only source of urinary glucose in fasting phlorhizin

[2]Similar changes in the non-protein R. Q. as calculated in the ordinary way have been recently observed by Boothby and Sandiford, but they did not note any diminution of efficiency following high fat, low carbohydrate diets (personal communication).

dogs was protein. If, as suggested by Krogh, fat may be converted to carbohydrate, and if this mechanism is not affected by phlorhizin, urinary glucose would be furnished from fat as well as protein, and the D:N ratios would not be fixed.

This objection may be answered by assuming that carbohydrate formed from fat is immediately oxidized. In that case there would be no alteration of the D:N ratios or of the respiratory quotients. This hypothesis carries the rather dangerous assumption that in a state of complete diabetes carbohydrate or a carbohydrate-like substance formed from fat is readily oxidizable at a time when the ordinary forms of carbohydrate cannot be utilized. Also the view that fat is oxidized as carbohydrate creates serious difficulties when one attempts to explain why ketone bodies arise from fat and not from carbohydrate unless one is prepared to make two assumptions; *viz.*, that the conversion of fat to carbohydrate occurs only during exercise, and that ketone production does not result when fat is oxidized during work. There are certain experimental data which indicate that the second assumption runs counter to the facts.

Sources of Energy in Muscular Exercise.

While it may be freely admitted that the possibility of lactic acid formation from fat has not been excluded, this assumption is not necessary to explain the facts. Whatever the chemical transformations may be, the respiratory quotients indicate that, in diabetes, the chief, or in some circumstances, perhaps the only, source of energy from oxidation is fat. At this point much confusion has arisen in the literature.

On the one hand, experiments performed on isolated muscle by Fletcher and Hopkins, Hill, Meyerhof, and Embden, indicate that the only chemical mechanism for muscular contraction is the formation of lactic acid from carbohydrate. This has led to a wide-spread belief that carbohydrate furnishes the only source of energy for exertion. This belief has been strengthened by some experiments of Meyerhof (28) in which he found a respiratory quotient of 1.00 during the contraction of isolated muscle. On the other hand, there are many experiments on the respiratory metabolism of the intact animal during exertion which indicate

that the energy for muscular exercise comes from various foodstuffs. To be sure, exercise in a normal, well fed man is accompanied by a quotient which approaches unity (29). When high fat diets (26, 30) or high protein diets (30) are given, however, the respiratory quotients indicate that the energy is derived from the foodstuffs which preponderate in the diet. In prolonged fasting (30) and in severe diabetes (31, 32) the energy may be derived almost entirely from fat. For example, in the experiments of Anderson and Lusk, a dog which had fasted 13 days and then ran 10 miles for more than 3 hours, showed during the last hour a non-protein respiratory quotient of 0.713 or the theoretic value for human fat as given by Zuntz.

These two classes of evidence: one, concerned with the experiments on isolated muscle; the other, with the respiratory metabolism of intact animals, are not necessarily contradictory. Muscular contraction consists of two phases: a contractile phase, which is accompanied by the conversion of some form of carbohydrate into lactic acid, and a recovery phase during which oxidation occurs while part or all of the lactic acid is resynthesized to glycogen. The chemical reaction of the contractile phase, while accompanied by the liberation of a measurable amount of energy, requires no oxygen for its accomplishment. It produces no CO_2. It has no effect on the respiratory quotient. In the steady state of exercise the quotient is a reflection only of the oxidative processes which occur during the recovery phase.

That the energy during the contractile phase is derived from the formation and neutralization of lactic acid is well established. The experiments described in this paper indicate that even under the most extreme conditions of phlorhizin diabetes and of glycogen depletion these reactions may account for all the energy transformations during this phase. It is equally well established that during the recovery phase, the energy arises from oxidation; but the foodstuffs which are oxidized to furnish this energy have not been definitely determined. Fletcher and Hopkins considered that the energy came from the oxidation of lactic acid, since they found in muscles, which recovered from fatigue in an atmosphere of nitrogen, a much greater accumulation of lactic acid than in muscles which recovered in an atmosphere of oxygen. Their interpretation was generally accepted until

Hill and Meyerhof demonstrated that the heat given off during the recovery phase was much less than would result from the oxidation of all the lactic acid which had been produced. They found that the major portion of this compound was reconverted to a precursor during the recovery phase. This later work offers an alternative explanation for the accumulation of lactic acid under anaerobic conditions. The reconversion of lactic acid to glycogen is an endothermic reaction; and will not occur unless energy is supplied from the external surroundings. In an atmosphere of oxygen, this is furnished by oxidations. In an atmosphere of nitrogen there are no oxidations, and, therefore, no available energy for the reconversion of lactic acid. The latter product accumulates, not necessarily because of a specific failure to oxidize lactic acid, but because of the general failure to oxidize any substance which may supply the energy needed for the reconversion. Whether, under certain conditions, a part of the lactic acid serves as the substance whose oxidation furnishes the requisite energy, is not at all clear. The high quotients obtained by Meyerhof upon excised frog's muscle, may support that interpretation. In the living animal, however, the respiratory quotients offer convincing evidence that according to the various conditions of the experiment, the energy may be derived from carbohydrate alone, from fat alone, or from a combination of the two in any proportion. In severe or complete diabetes, fat must furnish the chief, if not the only, source of energy during the recovery phase.

SUMMARY AND CONCLUSIONS.

1. The effect of strychnine hypersensitivity and convulsions has been studied in three groups of animals: (*a*) normal dogs, (*b*) dogs rendered completely diabetic with phlorhizin, and (*c*) dogs with complete phlorhizin diabetes to whom adrenalin was given in order to reduce the glycogen stores to their lowest level.

2. The exertion which is induced during strychnine hypersensitivity is accompanied by an accumulation of lactic acid in the blood which may be almost as great in the phlorhizinized as in the normal dog.

3. Even when the glycogen reserves of the body have been re-

duced to a very low level by the prolonged administration of phlorhizin and adrenalin, lactic acid may accumulate in the blood during the muscular exertion elicited in a period of strychnine sensitivity.

4. Lactic acid may be formed in an animal which has lost the power to oxidize carbohydrate. This is evidence that the defect in phlorhizin diabetes is not in the formation of lactic acid from carbohydrate. The failure to burn carbohydrate at a time when the animal can form lactic acid may be interpreted in two ways: (*a*) lactic acid *is* an intermediary in carbohydrate combustion. The defect in phlorhizin diabetes lies in its oxidation. Or (*b*) lactic acid *is not* an intermediary in carbohydrate combustion. It is only formed in the anoxidative breakdown of carbohydrate. This process is quite separate and distinct from the oxidation of carbohydrate.

5. In phlorhizinized, as well as in normal dogs, the accumulation of lactic acid is almost certainly associated with the muscular exertion. This is evidence that even in animals unable to oxidize carbohydrate the normal mechanism for muscular contraction may be employed.

6. The probable source of lactic acid in these experiments has been discussed. Even after treatment with adrenalin the body retains substances, like protein, which are known to yield lactic acid. While fat cannot be excluded as a source of lactic acid this assumption is not necessary to explain the facts.

7. The sources of energy in muscular exercise have been discussed and the following working hypothesis has been advanced: (*a*) That during the contractile phase of muscular activity energy is available because of the chemical reactions which result in the formation and neutralization of lactic acid. These reactions occur even during complete phlorhizin diabetes and glycogen depletion. (*b*) That during the recovery phase the lactic acid is reconverted to glycogen by means of energy derived from the processes of oxidation. The respiratory quotients indicate that according to the conditions of the experiment the energy may come from carbohydrate alone, from fat alone, or from any combination of the two. In phlorhizin diabetes fat must furnish the chief, if not the only, source of energy during the recovery phase.

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IS THERE A TOXIN IN THE BLOOD OF PARATHYROIDECTOMIZED DOGS?

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The promptness with which tetany appears after complete parathyroidectomy and its usually rapidly fatal course indicated, to the minds of many investigators, that the symptoms were due to the presence of a toxin, which was normally rendered innocuous in the parathyroid glands or, by the aid of a hormone derived from these, in some other organ. Ammonia (1, 2), carbamic acid (3), and guanidines (4, 5) are among the substances that have been suggested as the poison. The existence of carbamic acid at the reaction of the blood is extremely unlikely and probably quite impossible. Using improved methods, Carlson and Jacobson (2) were unable to confirm Jacobson's earlier report (1) of an increased concentration of ammonia in the blood of parathyroidectomized animals. The present writer (6) had previously been unable to detect any such increase. More recently, he (7) has shown that the urines of parathyroidectomized dogs do not contain appreciable quantities of guanidine, methylguanidine, or dimethlyguanidine and that, therefore, the hypothesis that guanidine intoxication is the cause of tetany cannot be accepted.

In 1898, Ver Ecke (8) observed a marked decrease in the excretion of phosphorus in the urine after thyroidectomy. This was found by Greenwald (6) to be due to the coincident parathyroidectomy and not at all to the removal of the thyroids. This phenomenon has now been observed in the writer's laboratory in more than 50 dogs. Tetany has never appeared when this retention of phosphorus was absent and the drop in the excretion of phosphorus has been observed in only one out of

the ten or more dogs that did not develop tetany after attempted removal of the parathyroids. It seems permissible to conclude that, in this animal, the deficiency in parathyroid function was sufficient to produce a retention of phosphorus for 1 day but that the remaining parathyroid tissue had been able to function adequately in time to prevent the actual onset of tetany.

This retention is a very striking phenomenon. The amount of phosphorus in the urine of the 24 hours following the operation may fall to less than 2 per cent of its previous value (for example, see Experiment 4). If, after parathyroid insufficiency had become established, there were no excretion of phosphate at all, this would indicate that complete insufficiency had been established within 2 per cent of 24 hours, or 29 minutes. But, at the end of the 24 hour period, the kidneys were excreting some phosphate, so that it seems that the consequences of failure of parathyroid function must, in such animals, be manifested immediately upon removal of the glands. However, it takes from 24 to 96 hours for these changes to become sufficiently great to produce tetany. But there is no evidence, whatever, that parathyroid secretion is stored elsewhere in the organism.

The retention of phosphorus is accompanied by an increase in the "acid-soluble" phosphorus of the blood and plasma (9). It was at first thought that tetany might be due to poisoning with phosphate or with inosinic acid, but appropriate experiments (9, 10, 11) showed that this was not the case.

By means of crossed circulation between normal and parathyroidectomized dogs or between one animal and the isolated limbs of another or by dialyzing normal and tetany bloods and then using these in perfusion experiments, MacCallum and Vogel (12) and MacCallum, Lambert, and Vogel (13) attempted to determine whether the tetany of parathyroidectomized dogs was due to the presence of a toxin or to the deficiency of calcium in the blood. Their results indicated, quite clearly, that it was the fall in the concentration of calcium in the blood that was the immediate cause of tetany, but they did not disprove the possibility that this decrease was not itself due to the presence of a toxin.

The observations of Dragstedt and Peacock (14), who found that dogs on a milk diet or one containing large quantities

of lactose and producing an aciduric intestinal flora did not develop tetany after thyroparathyroidectomy as regularly as did those on an ordinary meat diet, were considered by them as proving that tetany is due to absorption of toxic material formed by proteolytic bacteria in the intestine. According to them, the function of the parathyroid glands is to detoxicate this material, but, if its formation be prevented by a suitable diet, the presence of the glands is not necessary to life.

The protective action of a milk diet has long been known. But Salvesen (15) has recently shown that milk that has been deprived of most of its calcium by treatment with oxalate, yet not containing any oxalate, will not protect against tetany. It seems probable that the effect of changes in the diet is due entirely to changes in the amount and availability of the calcium contained therein. It is quite possible that a proteolytic intestinal flora impedes calcium absorption while an acid condition of the intestinal contents promotes it.

The usual argument of those who favor the hypothesis that a toxin is the cause of tetany has always been the remission of symptoms and decreased electric excitability observed after bleeding and injection of salt solution. But, as MacCallum (16) points out, a similar decreased excitability may be observed in normal dogs and is probably due to impaired nutrition of the muscle. So it is not particularly significant that Luckhardt and Rosenbloom (17) could prevent the appearance of tetany by the injection of salt solution. The effect might have been due to a general impairment of muscle function. MacCallum (16) suggests that it might have been due to a washing out of phosphate with consequent increase in the amount of available calcium.

Backman (18) found that the plasma and serum of parathyroidectomized rabbits did not differ from those of normal rabbits in their action on isolated intestine. But, since the spontaneous movements of the intestine as well as its response to epinephrine were the same in rabbits in tetany as in normal animals, these experiments cannot be understood to indicate the absence of a toxin in tetany blood. They simply mean that the disturbance, whatever its nature, does not affect the intestine.

Trendelenburg and Goebel (19) studied the action of blood from normal and parathyroidectomized cats upon the isolated frog heart. This did not contract as powerfully in the blood of cats in tetany as in that of normal cats. The same difference was observed in the solutions of the ash of the bloods. The contractions were increased if $CaCl_2$ was added to the tetany blood. But, again, these experiments do not disprove the existence of a toxin in tetany blood. They simply prove that the only change from the normal in such blood *that affects the isolated frog heart* is the lowered concentration of calcium.

That the serum of rabbits bled at the height of tetany, when injected into rats, lowered the metabolism, as was reported by Jino (20), is interesting but scarcely of significance as evidence of a toxin causing tetany. This condition is not characterized by a lowered metabolism. It is not surprising that serum taken from an animal in the height of tetany should contain substances not found in normal serum, nor that one or more of these should depress metabolism. But the presence of these substances is as likely to be an effect of tetany as its cause.

In order to indicate the presence of a toxin which produces tetany, it would seem to be necessary to show that the blood, or an extract prepared from it, will produce tetany-like symptoms in animals not previously showing such symptoms. The present paper deals with an unsuccessful attempt to demonstrate such action. Small thyroparathyroidectomized dogs were injected with extracts of blood from dogs in active tetany. In each case, the extract was prepared from a volume of blood much greater than that circulating in the body of the recipient. In this manner, it was hoped to avoid the criticism that any tetany that might develop was due to perfusion with a calcium-poor liquid. The recipient was left with all the calcium he previously had and, if tetany had appeared, the presence of a toxin would have been made very probable.

In order to avoid the possibility that the toxin might be neutralized by the parathyroids of the recipient or by parathyroid secretion circulating in the recipient, a complete thyroparathyroidectomy was performed on the day before the injection experiment. No animal was used unless the urine of the succeeding 24 hours showed the marked decrease that is characteristic

of a successful parathyroidectomy. The animal could, at the time of the injection of 28 to 32 hours after the thyroparathyroidectomy, be regarded as defenseless against the hypothetical toxin.

The injections were made, under cocaine anesthesia, into a femoral vein. In some of the experiments, samples of blood for analysis were drawn from the femoral artery of the other leg. The expected low content of calcium was always observed.

In the first experiment, the blood of the tetany dog was precipitated with sodium tungstate and hydrochloric acid. The tungstate-free filtrate was evaporated *in vacuo* at a temperature of not over 37° and injected. One convulsion was observed, but this was not like those of parathyroidectomized dogs and, upon analysis, the blood plasma was found to contain as much sodium as had been found in the plasma of dogs in convulsions after the injection of sodium chloride (10). The convulsions were evidently due to the large amount of sodium chloride injected.

A similar result was obtained in the next experiment. The tetany blood was precipitated with picric acid and the protein-free filtrate was evaporated *in vacuo* at a temperature not over 37°, acidified with hydrochloric acid, and extracted with benzene in a continuous extraction apparatus until free from picric acid. After neutralization with sodium hydroxide and further evaporation *in vacuo*, this was injected into a dog that was already showing some twitching following thyroparathyroidectomy. The first few cubic centimeters relieved the twitching and no further symptoms were observed until near the close of the injection when convulsions appeared. Again, the plasma was found to contain as high a concentration of sodium as in convulsions following the injection of sodium chloride.

These procedures having been found unsatisfactory, in the subsequent experiments, the blood was drawn directly into redistilled 95 per cent alcohol. About 4 volumes of alcohol to 1 of blood were used. The precipitate was filtered off on a Büchner funnel, washed with alcohol, and pressed dry. The combined filtrates were evaporated *in vacuo* at a temperature not over 35° until they foamed a great deal. The liquid was then made just acid to Congo red with hydrochloric acid and the lipoids were then precipitated with chloroform (21). After standing

overnight, the clear supernatant liquid was filtered, the precipitate was washed with acidulated chloroform-water, and the combined filtrate and wash liquids were neutralized with sodium hydroxide, evaporated at a temperature not over 35° and then kept on ice until required for the injection, when they were warmed to 40°. This material was never toxic. The dogs received, without apparent ill effect, extracts equivalent to amounts of blood at least twice as large as those circulating in their own bodies. The wounds were then closed and the development of tetany awaited. This never failed. It appeared from 12 to 36 hours later. In that manner, the control of the thoroughness of the parathyroidectomy was made complete. But there was no evidence that the development of tetany had been in any way accelerated by the injection.

The chemical treatment of the blood was rather mild. Any poisonous substance that was not volatile or very unstable should have been preserved unchanged and injected in all its potency. That no symptoms at all resembling tetany were observed indicates that this is not due to poisoning with relatively simple substances such as the guanidines. If there is a toxin in the blood after parathyroidectomy, this toxin must be either volatile, or quite insoluble in 80 per cent alcohol or else be extremely unstable.

In the author's opinion, there is no such toxin. The hypothesis is quite unnecessary and is not supported by any satisfactory evidence. A consideration of the pathogenesis of tetany is reserved for another communication.

Protocols.

The dogs were fed mixtures of beef heart, lard, and cracker meal in varying proportions, which were, however, constant for any given animal. Most of them received infusorial earth to keep the feces solid, but a few received bone ash. They were not catheterized, except at the time of the thyroparathyroidectomy and not all of them, even then. Therefore, the absolute amounts of nitrogen and phosphorus excreted in the urine vary considerably from day to day.

Nitrogen was determined by Kjeldahl and phosphorus by Briggs' modification of that of Bell and Doisy (22). Calcium and sodium were determined by the methods of Kramer and Tisdall (23).

Experiment 1.—Dog 5. Weight 18.8 kilos. Dec. 15. Thyroparathyroidectomy. Dec. 18. Tetany, exsanguinated. The blood was pre-

cipitated with sodium tungstate and hydrochloric acid. Filtrate equivalent to 506 gm. of blood was evaporated at a temperature not over 37° to small volume. pH 5.0. Tests for tungstic acid were negative. Injected into Dog 6.

Dog 6. Weight 5.3 kilos.

Date.	N	P	$\frac{P \times 100}{N}$	Remarks.
Dec. 26–27	10.06	0.956	9.50	Urine of nearly 2 days. Thyroparathyroidectomy, Dec. 27.
" 27–28	2.96	0.074	2.51	Only part of day's urine.
" 28				
4.30 p.m.				Serum calcium 8.21 mg. per 100 cc.
4.49–5.34 p.m.				Injected material equivalent to 300 cc. blood.
5.35 p.m.				Tonic-clonic convulsion, then quiet.
5.38–5.45 p.m.				Injected material equivalent to 100 cc. blood. Heart stopped. Blood from jugular vein contained 561 mg. sodium per 100 cc. plasma.

Experiment 2.—Dog 4. Weight 18 kilos. Dec. 10. Thyroparathyroidectomy. Dec. 12. Tetany, exsanguinated. The blood was precipitated with picric acid. Filtrate equivalent to 492 gm. of blood was evaporated at a temperature not over 37°, HCl was added, and the mixture extracted with benzene in a continuous extraction apparatus until free from picric acid. After neutralization and further evaporation at a temperature not over 37°, it was injected into Dog 8.

Dog 8. Weight 3.7 kilos.

Date.	N	P	$\frac{P \times 100}{N}$	Remarks.
Jan. 1–2	1.51	0.136	8.99	
" 2–3	1.92	0.068	3.58	Jan. 2, 9.30 a.m. Thyroparathyroidectomy.
" 3				
3.30 p.m.				Slight but unmistakable twitching, 7.54 mg. calcium per 100 cc. serum.
3.30–4.28 p.m.				Injected material equivalent to 492 gm. blood from Dog 4. Twitching disappeared within 15 min. of beginning injection. One convulsion when 0.8 gm. of the material had been injected (4.20 p.m.).
4.30 p.m.				Sodium content of plasma 594 mg. per 100 cc.

Experiment 3.—Dog 11. Weight 14.3 kilos. Jan. 24. Thyroparathyroidectomy. Jan. 30. Occasional but not continuous twitching to Feb. 5 when dog was seen in a severe attack of tetany and was exsanguinated at once. 798 gm. of blood were precipitated with alcohol. The filtrate and wash alcohol were evaporated at a temperature not over 35°, the lipoids were precipitated with hydrochloric acid and chloroform, and the filtrate was neutralized and again evaporated *in vacuo*. This was injected into Dog 14.

Dog 14. Weight 5.3 kilos.

Date.	N	P	$\frac{P \times 100}{N}$	Remarks.
Feb. 8– 9	1.36	0.166	12.2	
" 9–10	1.48	0.172	11.6	
" 10–11	1.32	0.167	12.5	
" 11–12	1.99	0.028	1.4	Thyroparathyroidectomy, Feb. 11.
" 12, 2 p.m.				Serum calcium 9.33 mg. per 100 cc. Injected material from Dog 11 in 11 min. No ill effect.
" 12–13	2.34	0.132	5.6	Quite normal.
" 13–14	2.18	0.024	1.1	Severe tetany, Feb. 14.

Experiment 4.—Dog 18. Weight 20.8 kilos. Apr. 16. Thyroparathyroidectomy. Apr. 18. Twitching which was more pronounced on Apr. 19, when the dog was bled during a severe attack. 1,340 gm. of blood were precipitated with alcohol and treated as described in Experiment 3.

Dog 22. Weight 2.9 kilos.

Date.	N	P	$\frac{P \times 100}{N}$	Remarks.
May 5–6	1.31	0.095	7.75	
" 6–7	1.42	0.114	8.04	
" 7–8	1.31	0.0018	0.14	Thyroparathyroidectomy, May 7.
" 8	0.29	0.0039	1.34	2 p.m. Serum calcium 8.34 mg. per 100 cc. Injected material equivalent to 1,220 gm. blood from Dog 18 in 25 min. No symptoms.
" 8–9	1.60	0.141	8.87	Dog did not eat food.
" 9–10	1.11	0.018	1.66	" " " " " Tetany doubtful.
" 10–11	0.81	0.043	5.31	Dog did not eat food. Tetany doubtful.
" 11–12	1.23	0.119	9.54	Dog did not eat food. Unmistakable tetany. Serum calcium 6.69 mg. per 100 cc.

Experiment 5.—Dog 21. Weight 17.9 kilos. Apr. 28. Thyroparathyroidectomy. May 1. Twitching. May 2. More pronounced tetany, exsanguinated. 818 gm. of blood precipitated with alcohol and treated as described in Experiment 3.

Dog 24. Weight 5.8 kilos.

Date.	N	P	$\frac{P \times 100}{N}$	Remarks.
May 19–20	2.55	0.160	6.30	
" 20–21	3.03	0.236	7.79	
" 21–22	3.36	0.026	0.76	Thyroparathyroidectomy, May 21. May 22, 1.30 p.m., serum calcium 8.18 mg. per 100 cc. Injected material equivalent to 750 gm. blood in 9 min. No ill effect.
" 22–23	2.86	0.134	4.68	Tetany, May 23. Serum calcium 7.38 mg. per 100 cc.

Experiment 6.—Dog 23. Weight 11.3 kilos. May 10. Thyroparathyroidectomy. May 12. Tetany. 791 gm. of blood precipitated with alcohol and treated as described in Experiment 3.

Dog 25. Weight 3.4 kilos.

Date.	N	P	$\frac{P \times 100}{N}$	Remarks.
May 17–18	1.62	0.138	8.45	
" 18–19	2.15	0.142	6.58	
" 19–20	1.58	0.014	0.91	Thyroparathyroidectomy, May 19. May 20, 2 p.m., serum calcium 9.25 mg. per 100 cc. Injected material equivalent to 700 gm. blood from Dog 23 in 18 min. No symptoms.
" 20–21	1.90	0.109	5.75	Normal.
" 21–22	2.40	0.157	6.54	Tetany, May 22.

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ALKALOSIS PRODUCED BY INJECTIONS OF HYDRAZINE SULFATE IN DOGS.*

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Sweet and one of us (1) observed a high CO_2-combining power of the blood in fasting Eck's fistula dogs after pancreatectomy. These animals when fasting, showed a very marked reduction in sugar excretion as compared with dogs upon which pancreatectomy alone had been performed. These observations have led us to suppose alkalosis may be associated with other forms of liver injury especially those accompanied by hypoglucemia. Underhill (2) has shown that hydrazine produces hypoglucemia when administered parenterally to fasting dogs, and Wells (3) demonstrated that hydrazine intoxication produces a very marked degeneration of the liver. Underhill and Baumann (4) noticed a decrease in the acidity of the urine of dogs after the injection of hydrazine.

We have studied the relation of hypoglucemia to the CO_2-combining power and pH of the blood of dogs poisoned with hydrazine and have attempted to find out what constituents of the blood are responsible for the increase in CO_2-combining power. The urine (mixed with vomitus) has been studied also in order to determine the loss of various acid and basic radicals which might account for the alkalosis we have observed.

Method.

In the first series of four dogs, we have investigated only the changes in sugar, CO_2-combining power, and pH of the blood.

*A preliminary report of these observations was presented at the St. Louis meeting of the American Society of Biological Chemists, December, 1923.

The remaining animals were used in an effort to determine what increase in basic ions or decrease in acid ions is responsible for the alkalosis which we have invariably found in dogs after hydrazine injection. The dogs, kept in large metabolism cages, were fasted for at least 24 hours before the injection and the fasting continued throughout the course of each experiment. 50 mg. of hydrazine sulfate, per kilo of body weight, were given as the initial dose and 25 mg. were given every 2nd day thereafter while the experiment was continued. Under this treatment the animals would live 2 or 3 weeks if the amount of blood drawn was not too great. In the second series of experiments, the animals died within relatively few days, apparently because they did not endure the relatively large loss of blood which was required for this part of the work. Whenever the urine was saved for analysis, it was preserved under toluene.

Analytical Methods.

The CO_2-combining power of the blood was determined by the method of Van Slyke and Cullen (5) and the pH of the blood by Cullen's (6) method. In the first series of experiments, MacLean's (7) method for blood sugar was used while the Shaffer-Hartmann (8) method was used in the second series. Our method for the determination of ammonia in the blood was essentially that of Nash and Benedict (9). The sodium of the blood was determined by the method of Kramer and Gittleman (10) and the chlorine by Van Slyke's (11) method. The usual methods were used in analyzing the urine except that Tisdall and Kramer's (12) was used for urinary potassium.

RESULTS.

The experiments on Dogs 1 to 4 demonstrate very distinctly that alkalosis, as shown by the increase in pH and CO_2-combining power of the blood, follows after hydrazine injections. In the case of Dog 1, the pH values given (see tables) are probably too low, but the variations among the determinations are accurate as the readings were made against the same phosphate standards. Later experiments indicate that this phosphate solution was slightly too alkaline. There can be no doubt, however, that these

TABLE I.

Dog 1. Male. Weight 8.9 kilos.

Date.	pH	CO_2	Blood sugar.	Remarks.
1923		*vol. per cent*	*mg. per 100 cc.*	
June 4	7.13	48.09		Normal. Injected 445 mg.
" 5	7.27	57.17		
" 6	7.29	57.57	89.0	Injected 200 mg.
" 7	7.36	55.12	60.6	
" 8	7.43	57.57	49.0	" 200 "
" 9	7.29	65.51	55.4	
" 10	7.29	64.33	54.2	" 200 "
" 11	7.34	64.24	54.2	
" 12	7.50	65.39	52.0	
" 14	7.38	65.54	67.5	" 200 "
" 15		65.75	67.5	" 200 "
" 16	7.39	73.71	37.5	
" 17	7.39	69.11	67.8	
" 18	7.34	67.76	75.0	" 200 "
" 19	7.49	73.68	64.2	
" 20		63.21	31.9	
" 21		59.81		" 200 "
" 22	7.39	69.06	82.5	
" 23		69.03	66.0	" 200 "
" 24				Animal found dead.

TABLE II.

Dog 2. Male. Weight 7.5 kilos.

Date.	pH	CO_2	Blood sugar.	Remarks.
1923		*vol. per cent*	*mg. per 100 cc.*	
Oct. 23	7.26	52.63	74	Normal. Injected 300 mg.
" 24	7.35	60.04	94	
" 25	7.32	62.13	69	Injected 150 mg.
" 26	7.23	47.48	80	
" 27	7.23	54.30	65	" 200 "
" 29	7.37	61.28	65	
" 30	7.47	69.85	78	Arterial blood.
" 31	7.44	75.07	59	" "
				Animal died after puncture of heart.

TABLE III.

Dog 3. Female. Weight 8.4 kilos.

Date.	pH	CO_2	Blood sugar.	Remarks.
1923		*vol. per cent*	*mg. per 100 cc.*	
Nov. 2	7.30	50.51		Normal.
" 3	7.31	50.63	75.0	" Injected 400 mg.
" 5	7.58	54.23	87.0	Injected 200 mg.
" 6	7.27	58.54	75.0	
" 7	7.44	60.28	59.0	" 200 "
" 8	7.57	58.84	75.0	
" 9	7.52	56.90	61.0	" 200 "
" 10	7.63	63.85	59.2	
" 12		62.63	53.0	" 200 "
" 13	7.64	61.72	73.0	
" 14	7.53	63.96	58.5	" 300 "
" 15	7.43	63.35	73.0	
" 16				" 400 "
" 17	7.51	48.50	75.0	Animal killed by ether. All samples arterial blood.

TABLE IV.

Dog 4. Male. Weight 9.66 kilos.

Date.	pH	CO_2	Blood sugar.	Remarks.
1923		*vol. per cent*	*mg. per 100 cc.*	
Nov. 19	7.35	43.86	80	Normal. Injected 900 mg.
" 20	7.42	46.15	87	
" 21	7.45	52.10	71	
" 22	7.40	58.92	87	
" 23	7.55	52.85	98	
" 24	7.36	51.44	74	
" 26	7.58	56.64	66	
" 27	7.41	57.30	61	
" 28	7.48	57.17	64	

pH results are entirely satisfactory for comparative purposes. This dog showed a maximum increase in the pH of the plasma of 0.34. This increase did not appear, however, until after several injections of hydrazine sulfate. The other dogs in this series did

not show quite so marked an increase in pH, but in every case it was unmistakable.

The increase in CO_2-combining power in the animals of this series of experiments varied from 25.62 volumes per cent in No. 1, to 13.45 volumes per cent in No. 3. In the case of No. 1, the increase was more than 10 millimol equivalents per liter of whole blood. In No. 3, which showed the least increase, it amounted to more than 5 millimol equivalents per liter.

The relation of the hypoglucemia to the alkalosis was only fairly definite. The smallest amount of blood sugar was usually found when the degree of alkalosis was the greatest. The relationship between the sugar and the CO_2-combining power of the blood seemed more constant than the relation of the pH to the blood sugar. With few exceptions, the hypoglucemia was most marked when the alkalosis was the severest and whenever the CO_2-combining power of the blood decreased, the blood sugar began to return to normal. The hypoglucemia never appeared until after there was a definite increase in both pH and CO_2-combining power of the blood. A certain degree of alkalosis always occurred within the first 24 hours, while the hypoglucemia developed considerably later, being noticeable 48 hours after the first injection, or even later. This clearly indicates that the first noticeable effect of hydrazine intoxication on the blood is the production of alkalosis. This fact points to the possibility of a causal relation of the alkalosis to the hypoglucemia of hydrazine intoxication.

The observation of Hahn, Massen, Nencki, and Pawlow (13) that carbamates may appear in the urine of Eck's fistula dogs, and the older statements that an increased excretion of ammonia may occur in cases of phosphorus poisoning, cirrhosis, and acute yellow atrophy of the liver, pointed to the possibility that an increased ammonia content of the blood might be a causal factor in producing the observed alkalosis.

The experiment upon Dog 5 convinced us that the ammonia of the blood could not play more than an insignificant rôle in producing the alkalosis. Although the CO_2-combining power of the blood increased 28.27 volumes per cent after the administration of hydrazine, the ammonia only increased 0.21 mg. per 100 cc. or less than 1 per cent of the amount required to give a CO_2-combining power proportional to the observed increase.

In all our other experiments, we have studied the molecular ratio of sodium to chlorine in the blood and also the elimination of acid, phosphate, chlorine, ammonia, and potassium in the urine and vomitus. No very serious attempt was made to keep the urine and vomitus separate at this time as we have been interested for the present in the total elimination. For the sake of brevity, we will confine our discussion to the results obtained within 24 hours after the injections of hydrazine sulfate, although all our experiments were continued for a somewhat longer period.

Dogs 6 to 12 show an increase of sodium in proportion to chlorine in the serum after the injection. The average increase in the value of the Na:Cl ratio the day after the injection was 0.122; the least increase, in Dog 11, was 0.014; and the greatest, in Dog

TABLE V.

Dog 5. Male. Weight 7.94 kilos.

Date.	CO_2	Ammonia.	Remarks.
1924	*vol. per cent*	*mg. N per 100 cc.*	
Mar. 24	47.48	0.05–0.1	Normal. Injected 400 mg.
" 25	61.87	0.1	
" 26	62.21	0.06	Injected 200 mg.
" 27	60.67	0.1	" 200 " on Mar. 28.
" 29	64.80		
" 31	68.30	0.18	" 200 "
Apr. 1	73.90	0.26	
" 2	75.75	0.16	

7, was 0.202. This increase was usually but not uniformly maintained throughout the course of the experiment. Variations in the concentration of serum proteins must, of necessity, affect the ratio of sodium to chlorine. No attempts have been made to follow the variations in serum proteins. Some other acid radicals may have been retained along with the excess of sodium, but this seems improbable because there was no evidence of impairment of the kidney function. In some cases the amount of sodium in the serum after the injections was greater than the normal, but the chlorine in the serum was always decreased. It appears, therefore, that the observed alkalosis was produced by the elimination of chlorine or some other acid radical, possibly phosphate, in excess of sodium.

TABLE VI.

Dog 6. Male. Weight 16.2 kilos.

Date.	Blood analyses.				Urine analyses (24 hr. urines).				Remarks.
	CO_2	Cl	Na	Na:Cl	pH	Acid 0.1 N.	Cl	P_2O_5	
1924	*vol. per cent*	*m.-Eq. per l.*	*m.-Eq. per l.*			*cc.*	*gm.*	*gm.*	
Apr. 10	49.98	105.2	113.2	1.077					Normal. Injected 800 mg.
" 11	64.43	108.8	137.3	1.262	5.8	246.4	1.850	1.185	
" 12	72.76	104.0	133.6	1.285	6.4	178.5	0.150	1.220	Injected 400 mg.
" 13					5.8	217.6	0.558	2.282	
" 14	56.50	97.8	133.6	1.366	6.7	232.4	0.149	2.036	" 400 "

TABLE VII.

Dog 7. Female. Weight 8.4 kilos.

Date.	Blood analyses.				Urine analyses (24 hr. urines).				Remarks.
	CO_2	Cl	Na	Na:Cl	pH	Acid 0.1 N.	Cl	P_2O_5	
1924	*vol. per cent*	*m.-Eq. per l.*	*m.-Eq. per l.*			*cc.*	*gm.*	*gm.*	
Apr. 18	47.43	105.8	112.1	1.067	6.0	113.0	0.336	0.5770	Normal. Injected 400 mg.
" 19	57.18	105.0	133.2	1.269	5.3	189.0	1.176	1.0030	
" 20									
" 21	62.75	101.8	132.4	1.303	6.4	97.2	1.080	1.0810	Injected 200 mg.
" 22					6.0	330.7	0.323	3.0065	" 200 "
" 23	64.67	95.8	123.9	1.295	6.4	6.2	0.345	0.0568	
" 24	68.66	94.8	109.7	1.157	6.4	177.1	0.014	1.2560	" 200 "
" 25	70.83	90.4	116.6	1.288	6.7	102.0	0.180	0.8190	
" 26	69.08	83.8	117.7	1.404			0.008	0.4230	" 200 "
" 28	68.93	90.6	117.7	1.329	8.2	25.0	0.016	0.5090	
" 29	66.95	89.2	97.1	1.089			0.030	0.1010	" 200 "
" 30	71.73	88.0	101.8	1.157	7.6	12.5		0.6480	

The increase in acid elimination after the injection of hydrazine in Dogs 8, 9, and 10, was particularly marked. It can be shown that the amount of acid lost was greater than the increase in alkali in the blood, except in the case of Dog 12. In this animal,

TABLE VIII.

Dog 8. Female. Weight 21.1 kilos.

Date.	Blood analyses.					Urine analyses (24 hr. urines).				Remarks.
	CO_2	Cl	Na	Na:Cl	pH	pH	Acid 0.1 N.	Cl	P_2O_5	
1924	*vol. per cent*	*m.-Eq. per l.*	*m.-Eq. per l.*				*cc.*	*gm.*	*gm.*	
Apr. 18	47.43	107.4	130.3	1.214	7.41	5.8	201.6	0.781	1.003	Normal. Injected 1,055 mg.
" 19	59.11	104.8	131.3	1.253	7.41	5.0	618.8	3.300	3.128	
" 20						5.0	86.0	0.288	0.409	Injected 527 gm.
" 21	62.75	99.0	125.8	1.271	7.65	6.4	383.4	0.967	2.331	
" 22						5.8	220.4	0.922	1.601	" 527 "

TABLE IX.

Dog 9. Female. Weight 6.65 kilos.

Date.	Blood analyses.					Urine analyses (24 hr. urines).				Remarks.
	CO_2	Cl	Na	Na:Cl	pH	Acid 0.1 N.	Cl	P_2O_5	NH_3-N	
1924	*vol. per cent*	*m.-Eq. per l.*	*m.-Eq. per l.*			*cc.*	*gm.*	*gm.*	*gm.*	
Apr. 29	46.74	116.0	139.7	1.204	7.2	36.0	0.252	0.341	0.0798	Normal. Injected 332 mg.
" 30	54.91	103.4	139.7	1.351	5.7	149.5	1.170	0.749	0.1397	
May 1	55.56	102.4	123.0	1.019	6.4	46.3	0.195	0.341	0.0130	Injected 200 mg.

TABLE X.

Dog 10. Female. Weight 8.6 kilos.

Date.	Blood analyses.					Urine analyses (24 hr. urines).					Remarks.
	CO_2	Cl	Na	Na:Cl	Blood sugar.	pH	Acid 0.1 N.	Cl	P_2O_5	NH_3-N	
1924	*vol. per cent*	*m.-Eq. per l.*	*m.-Eq. per l.*		*mg. per 100 cc.*		*cc.*	*gm.*	*gm.*	*gm.*	
May 2	47.15	122.8	127.3	1.037	91	6.3	155.0	0.390	0.563	0.217	Normal. Injected 430 mg.
" 3	48.76	120.0	143.4	1.223	145	5.7	279.6	0.652	1.039	0.634	

TABLE XI.

Dog 11. Female. Weight 7.62 kilos.

Date.	Blood analyses.					Urine analyses (24 hr. urines).						Remarks.
	CO_2	Cl	Na	Na: Cl	Blood sugar.	pH	Acid 0.1 N.	Cl	P_2O_5	NH_3-N	K	
1924	*vol. per cent*	*m.-Eq. per l.*	*m.-Eq. per l.*		*mg. per 100 cc.*		*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	
May 5	45.31	114.4	127.6	1.116	80	6.8	99.0	0.216	0.887	0.2268	0.4510	Normal. Injected 381 mg.
" 6	51.07	109.0	123.1	1.130	109	5.2	159.0	1.280	0.955	0.2192	1.0465	
" 7	54.97	107.3	125.5	1.170	98	6.4	97.5	1.326	1.479	0.1820	3.7600	Injected 160 mg.

TABLE XII.

Dog 12. Male. Weight 26.36 kilos.

Date.	Blood analyses.					Urine analyses (24 hr. urines).						Remarks.
	CO_2	Cl	Na	Na: Cl	Blood sugar.	pH	Acid 0.1 N.	Cl	P_2O_5	NH_3-N	K	
1924	*vol. per cent*	*m.-Eq. per l.*	*m.-Eq. per l.*		*mg. per 100 cc.*		*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	
May 8	45.94	106.0	119.5	1.127	83	7.2	98	0.588	0.8599	0.1372	0.1013	Normal. Injected 1.318 gm.
" 9	57.80	105.2	127 3	1.210	106	5.0	210	1.750	0.9100	0.3360	1.3820	
" 10	54.55	102.4	119.5	1.167	103	6.8	210	0.900	1.2620	0.7875(?)	0.2890	Injected 659 mg.

the increase in CO_2-combining power of the blood is much greater than the acid eliminated. The acid eliminated was equivalent to only 224 cc. of 0.05 N acid, while the gain in base by the blood was approximately equivalent to 562.8 cc. of 0.05 N alkali. Even when the excess ammonia excretion was taken into account, the acid loss was equivalent to 509.6 cc. of 0.05 N solution. It is to be noted further that the chlorine excreted in excess of the normal dog is equivalent to 644 cc. of 0.05 N solution. Since there was an actual increase in sodium in the serum of this dog, it would seem that some other source of sodium was available. It is possible that the chlorine was excreted in association with some of the excess potassium on this day and sodium from the tissues (presumably the liver) remained in the blood and organs of the body.

Loss of acid through the stomach undoubtedly accounts for a large portion of the alkalosis found in these animals, but we are not convinced that this is the only factor involved. Cushny (14) and later Sanders and one of us (15) have shown that basic phosphates injected parenterally are excreted largely as acid phosphates in the urine. The large excretion of phosphate after the administration of hydrazine sulfate might account for a portion of the increase in bases in the blood. Those cases (Dogs 10 and 12) in which there was an actual increase in the sodium of the serum might be explained in this fashion.

SUMMARY.

Alkalosis, as shown by an increase in pH of the plasma and the CO_2-combining power of the whole blood, develops within 24 hours of the injection of 50 mg. of hydrazine sulfate per kilo of body weight into fasting dogs.

The alkalosis appears earlier than the hypoglucemia, but no causal relation between them has been demonstrated.

In one experiment, a very slight increase of blood ammonia was found. This increase was not sufficient to account for any significant portion of the increased CO_2-combining power of the blood.

The ratio Na:Cl of the serum is increased in hydrazine intoxication. This probably accounts for the alkalosis.

The loss of acid from the stomach is undoubtedly responsible for the depletion in the blood of chlorine ions.

The suggestion is made that a portion of the excess sodium of the serum may be due to the excretion of the phosphates in a more acid form than they exist in the blood.

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THE DETERMINATION OF NITROGEN IN CONNECTION WITH THE WET COMBUSTION METHOD FOR CARBON.*

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In the study of the carbon metabolism of *Fusarium lini* on glucose solutions[1] the wet combustion method of Rogers and Rogers,[2] as modified by Gortner,[3] was found very satisfactory for determining carbon in the mycelium. Since in work of this kind two cultures grown under identical conditions often show great variations in the nature of their growths, it is desirable to make as many determinations as possible on the same sample. In connection with the above mentioned study it was desirable to determine nitrogen as well as carbon in the mycelium. Due to the fact that only a few tenths of a gram of material were available in each case, it occurred to the authors that the residue from the digestion with chromic acid might be used for the determination of nitrogen by simply making alkaline and distilling the ammonia as in the usual Kjeldahl procedure.

To test this method wet combustions were run on asparagine, digesting with 50 cc. of carbon dioxide-free water, 5 gm. of potassium dichromate, and 75 cc. of concentrated sulfuric acid, at a temperature just below the boiling point for about $2\frac{1}{2}$ hours or until aeration was complete. Because of the large amount of acid to neutralize, the residue was divided, approximately equal amounts

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[3] Gortner, R. A., *Soil Sc.*, 1916, ii, 395.

being placed in two large Kjeldahl flasks, diluted, made alkaline with sodium hydroxide solution, and the ammonia from both flasks distilled into the same standard acid solution. Table I indicates practically theoretical results on asparagine which show that both amide and amino nitrogen may be determined in this manner.

Since the method was to be used on fungus mycelium the next thing to consider was its application to such a material. Since no large amount of *Fusarium lini* mycelium was available a quantity of puffballs, another fungus material, was gathered, dried, and submitted to analysis. As indicated in Table I, lower results were obtained by the wet combustion method than by the Kjeldahl-Gunning method. At first these low results were attributed to incomplete oxidation of the material, consequently several modifications of the procedure were tried, such as varying the time of heating, and the amounts of acid and potassium dichromate used. No modification gave higher returns of nitrogen than the usual wet combustion method. Since the wet combustion method gave uniformly low results for nitrogen it was thought that a factor might be worked out which could be used to convert these results into true nitrogen values as determined by the Kjeldahl method. For this purpose dried *Fusarium lini* mycelium was used. In this case the wet combustion nitrogen values agreed exceedingly well with those obtained by the Kjeldahl method (see Table I). Apparently the puffballs contained some material which was interfering with the nitrogen determination by the wet combustion method. If chlorides were present the reaction represented by the following equation might be taking place, thus accounting for the loss of nitrogen:

$$K_2Cr_2O_7 + 2NH_4Cl = 2KCl + Cr_2O_3 + 4H_2O + N_2$$

A qualitative test for chlorides in the dried puffballs proved to be strongly positive. The *Fusarium lini* mycelium being grown on a chloride-free medium contained no chlorides. To settle this question asparagine was analyzed again in the presence of varying amounts of hydrochloric acid. The results in Table I show plainly a loss of nitrogen. To show the application of this method to other materials, analyses were made of casein and gelatin, both of which were chloride-free. The results agreed well

with those obtained by the Kjeldahl method. The addition of sodium chloride to these materials lowered the nitrogen by the wet combustion method. On the other hand, egg albumin,

TABLE I.

Comparison of Kjeldahl and Wet Combustion Method for Determining Nitrogen.

Method.	Carbon.	Nitrogen.	
		Wet combustion.	Kjeldahl.
Asparagine.			
	per cent	*per cent*	*per cent*
Theoretical	31.98	18.65	18.65
Usual wet combustion	32.19	18.43	18.48
	32.19	18.68	18.61
	31.95	18.66	
" " " + 1 cc. 0.1 N HCl	32.16	17.86	
" " " + 5 " 0.1 N "	32.17	17.30	
" " " + 25 " 0.1 N "	32.28	15.25	
Dried puffballs (chlorides present).			
Usual wet combustion	40.64	7.56	8.62
	41.22	7.42	8.61
	40.54	7.42	8.64
Aeration method	40.82	8.54	8.58
	40.76	8.56	8.62
Dried *Fusarium lini* mycelium (chloride-free).			
Usual wet combustion	46.26	3.46	3.44
	46.76	3.54	3.48
Casein (chloride-free).			
Usual wet combustion	50.81	12.37	12.25
	50.77	12.29	12.35
	50.72	12.44	12.31
" " " + 0.1 gm. NaCl	50.72	7.84	
	50.79	8.23	
Aeration method in presence of 0.1 gm. NaCl	50.87	12.48	
	50.61	12.49	
	50.72	12.45	

TABLE I—*Concluded.*

Method.	Carbon.	Nitrogen.	
		Wet combustion.	Kjeldahl.
Gelatin (chloride-free).			
	per cent	*per cent*	*per cent*
Usual wet combustion	42.54	15.63	15.59
	42.78	15.80	15.75
	42.75	15.86	15.70
" " " + 0.1 gm. NaCl	42.71	8.56	
	42.65	9.01	
Aeration method in presence of 0.1 gm. NaCl	42.43	15.77	
	42.67	15.84	
Egg albumin (chlorides present).			
Usual wet combustion	43.82	11.82	12.86
	43.70	11.66	12.82
Aeration method	43.91	12.49	12.53
	43.84	12.45	12.37
	43.75	12.69	

which contained chlorides, gave lower results by the wet combustion method than by the Kjeldahl method, showing similar results to those obtained on puffballs.

It is evident from the above that chlorides interfere with the determination of nitrogen by the wet combustion method. Therefore, in order to make the method of value for biological materials a simple method must be devised for removing chlorides. Two methods were tried, one of which proved satisfactory. The unsuccessful attempt was made by conducting the oxidation in the presence of silver sulfate. The results were no higher than when no silver was present. The successful method consisted of an aeration of the sample in the presence of concentrated sulfuric acid before the addition of the potassium dichromate. The liberated hydrochloric acid was absorbed in a U-tube containing a saturated solution of silver sulfate. The sample was placed in the digestion flask, 50 cc. of concentrated sulfuric acid were added, aspiration was started, and the mixture heated to just below the boiling point. The time of aeration depends upon the amount of chlorides present, for the average sample a period of 30 minutes

is sufficient. With a 0.2 gm. sample to which 0.1 gm. of NaCl had been added, the chlorides were completely removed in 45 minutes. Heating is necessary for the rapid removal of chlorides, the charring which occurs does not interfere with the carbon determination because the apparatus remains intact during the entire procedure. Without heating all the chlorides could not be removed from a sample containing 0.1 gm. of NaCl in 6 hours. After the aeration was completed, 75 cc. of a solution containing 5 gm. of potassium dichromate dissolved in 25 cc. of concentrated sulfuric acid and 50 cc. of carbon dioxide-free water were placed in the separatory funnel and slowly run into the combustion flask. By this modification there was no danger of loss of carbon dioxide by opening the flask to add solid potassium dichromate. The determination was then continued as in the usual wet combustion method.

An examination of Table I indicates that the aeration method is very satisfactory for removing the interfering chlorides. The nitrogen values obtained on puffballs and egg albumin, which contain chlorides, after aeration, correspond with those obtained by the Kjeldahl method. Sodium chloride added to casein and gelatin may be removed by aeration and the results agree with those obtained on the chloride-free material. It should also be noted that the treatment given the sample in the aeration method does not alter the carbon values.

The writers feel that the wet combustion method for determining nitrogen will prove of value where it is desirable to determine both nitrogen and carbon on the same sample. It is believed that the method will give good results on any material to which the Kjeldahl method is applicable. When chlorides are present it is necessary to aerate the sample in the presence of hot sulfuric acid before adding the potassium dichromate.

A MODIFICATION OF THE MOLYBDIC METHOD FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN SERUM.

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(From the Huntington Fund for Cancer Research, Memorial Hospital, New York.)

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In 1914, Taylor and Miller (1) described a method for the determination of phosphates which depended on the precipitation of ammonium phosphomolybdate and the subsequent colorimetric determination of the molybdenum in the precipitate. Bell and Doisy (2) found that by reducing molybdic acid with a phenol (hydroquinone) in carbonate-sulfite solution, a blue color was obtained which was proportional to the amount of phosphorus present. Because of the rapid fading of the alkaline blue solution, Briggs (3) suggested that the reading be made in acid solution. Blood filtrates may become turbid and the color is relatively very faint.

Consequently an attempt was made in the present work to devise a procedure which would avoid turbid solutions and would yield a more intense and stable color. We have also combined the hydroquinone and bisulfite into one solution which keeps excellently.

The preparation of the reagent is as follows: To 20 gm. of pure molybdic acid (MoO_3)[1] in a flask add 25 cc. of 20 per cent sodium hydroxide solution and gently warm until the molybdic acid dissolves. Cool and dilute to 250 cc. Filter if necessary. A small quantity of this reagent (enough for a few days use) is diluted 1:1 with concentrated sulfuric acid as it is needed.

Determinations in blood serum are done in the following manner: 2 cc. of serum are diluted with a little water in a volumetric

[1] The molybdic acid used should be strictly pure and free from ammonia. The "Special" molybdic acid of Eimer and Amend is very satisfactory.

flask. 4 cc. of 20 per cent trichloroacetic acid are added and the contents of the flask made up to 10 cc. with water. Leave standing for at least 10 minutes and then filter through ashless filter paper. Place 5 cc. of the filtrate in a tube, add 3 cc. of water,[2] 1 cc. of the molybdic acid reagent (which has been diluted 1:1 with concentrated sulfuric acid), and 1 cc. of a solution containing 15 gm. of sodium bisulfite and 0.5 gm. of hydroquinone in 100 cc. Mix, stopper loosely, and place in a boiling water bath with a simultaneously prepared standard solution of potassium biphosphate containing 0.025 mg. of phosphorus in 5 cc. which has been treated similarly to the blood filtrate. This standard is prepared from a stock solution (containing 0.4394 gm. of dry potassium biphosphate in 1 liter of water and preserved with chloroform) by diluting 5 cc. of stock solution to 100 cc. with water and preserving with chloroform.

Standard phosphate solutions when determined according to these directions, using 0.025 mg. of phosphorus as standard, show a satisfactory degree of accuracy for phosphorus contents between 0.05 and 0.0125 mg.

The color produced is about three times as intense as that obtained by the Briggs procedure. The color of a standard solution has remained unchanged for several days. Longer heating than 10 minutes will produce some increase in color in both standard and blood filtrate, but the proportionality is the same at the end of 10 minutes as at the end of an hour. In twelve cases where phosphates were added to serum, an average of 103 per cent was recovered.

The results obtained by the use of this method are compared in Table I with the results obtained by the Briggs method. The figures are quite similar by both procedures even when widely varying amounts of phosphorus are present.

The method is not applicable to whole blood or to the serum or plasma from badly hemolyzed blood. This is due to the fact that the heating and strong acid will cause hydrolysis of the organic phosphorus compounds contained in the corpuscles.

[2] This procedure may be slightly modified by adding 3 cc. of 16 per cent sulfuric acid to the filtrate and 1 cc. of the molybdic acid reagent which has been diluted 1:1 with water instead of the concentrated sulfuric acid. The rest of the procedure is the same.

TABLE I.

Comparison of Proposed Method with Briggs' Method.

No.	Briggs' method.	New method.
	mg. P per 100 cc.	*mg. P per 100 cc.*
1	2.9	3.1
2	3.0	3.0
3	3.9	3.5
4	3.8	4.6
5	3.7	3.6
6	4.1	3.9
7	3.4	3.4
8	13.7	13.3
9	3.9	3.7
10	5.0	5.5
11	3.4	3.9
12	3.5	3.3
13	3.1	3.1
14	3.7	3.7
15	2.7	2.7
16	3.9	3.9
17	3.1	3.6
18	3.5	4.1
19	3.6	3.5
20	4.9	4.9
21	1.3	1.3
22	3.3	3.3

TABLE II.

Inorganic and Acid-Soluble P in Serum.

No.	Inorganic P.	Acid-soluble P.
	mg. per 100 cc.	*mg. per 100 cc.*
1	4.2	4.2
2	3.1	3.2
3	3.4	3.0
4	4.0	4.3
5	3.3	3.2
6	4.9	4.6
7	3.5	3.5
8	4.2	4.2
9	2.7	3.3

It may be noted that since in the technique described, the colors read are very much stronger than in the other phosphomolybdic acid methods, it is possible, when the blood available is quite limited, to use considerably less blood filtrate than is called for in our directions.

In agreement with Buell (4) we have failed to find any acid-soluble organic phosphorus in serum after hydrolysis with concentrated sulfuric acid. 5 cc. of the trichloroacetic acid filtrate digested until colorless with 0.6 cc. of concentrated sulfuric acid failed to show higher phosphate values than prior to such hydrolysis.

These figures are given in Table II.

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THE DETERMINATION OF PHENOLS IN THE BLOOD.

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In 1918, the present writers (1) described a method for the determination of phenols in the blood. Since then two additional methods have been published for phenol determination in blood—one by Pelkan (2) in 1922 and one by Rakestraw (3) in 1923. Both of these methods employ the Folin-Wu filtrate and the Folin-Denis phenol reagent. Pelkan removes uric acid as Folin and Denis do from the urine and determines phenols in the filtrate. Rakestraw precipitates uric acid by the Morris-Macleod zinc method prior to determination of phenols. Of these procedures, the Rakestraw is the more satisfactory because the final solution does not constantly become turbid as in the Pelkan method.

It is well known that the Folin-Denis phenol reagent is not specific for phenol bodies. For this reason we have deemed it desirable to attempt the determination of phenols in the blood by a procedure employing a different type of reagent from that which has heretofore been used, and one which is more specific for phenols than is the phosphotungstic-molybdic reagent. Such a reagent might offer the additional advantage that preliminary removal of the uric acid could be omitted.

The reaction of phenols with diazotized compounds has long been recognized. Moir (4) describes a sensitive test for phenols using diazotized *p*-nitroaniline base. We have employed this reagent to the quantitative colorimetric determination of phenols in the blood. The reagent is prepared by dissolving 1.5 gm. of *p*-nitroaniline base in 500 cc. of water with 40 cc. of concentrated hydrochloric acid. 25 cc. of this solution will be diazotized by 0.75 cc. of 10 per cent sodium nitrite solution. The diazotized reagent does not keep longer than 1 day, so that the nitrite should be added only to small quantities of the solution of the base.

The reagent thus prepared will yield an orange to red coloration with phenols, and the reaction is sufficiently delicate to be employed for phenol determination in blood filtrates. It is, however, necessary to dilute the blood only 1 to 5 instead of 1:10 as in the regular Folin-Wu procedure. As in the case of the Folin-Denis reagent, the nitroaniline reagent tends to become turbid with blood filtrates, but with the latter reagent this can be prevented by the addition of a colloid (gum acacia). The reaction takes place in solutions of very weak acidity. This condition is secured by the addition of sodium acetate prior to adding the reagent. Uric acid reacts so feebly with the nitroaniline reagent (giving about one-twentieth as much color as an equal weight of phenol) that removal of the uric acid is unnecessary. In no blood so far examined have we found any difference in the result of the preliminary precipitation of the uric acid.

The method, as we have employed it, is as follows: The blood is precipitated as in the Folin-Wu procedure save that 2 volumes of water are added instead of the 7 volumes usually used. To 10 cc. of the 1:5 filtrate are added 1 cc. of 1 per cent gum acacia solution, 1 cc. of 50 per cent sodium acetate solution, and 1 cc. of the diazotized nitroaniline reagent. After 1 minute, 2 cc. of 20 per cent sodium carbonate solution are added. A bright orange color is produced which should be read against a similarly treated standard phenol solution containing 0.025 mg. of phenol in 10 cc. The stock phenol solution is made according to the directions of Folin and Denis (5) as follows: Make a phenol solution in 0.1 N hydrochloric acid that contains about 1 mg. per cc. of solution. Transfer 25 cc. of this solution to a 250 cc. flask, add 50 cc. of 0.1 N sodium hydroxide, heat to 65°C., add 25 cc. of 0.1 N iodine solution, stopper the flask, and leave at room temperature for half an hour. Then add 5 cc. of concentrated hydrochloric acid and titrate the excess of iodine with 0.1 N sodium thiosulfate. Each cubic centimeter of 0.1 N iodine corresponds to 1.567 mg. of phenol. Dilute a portion of the solution so that 1 cc. contains 0.1 mg. of phenol. This dilution is made every few weeks and the final dilution (10 cc. = 0.025 mg.) is made daily.

The 0.025 mg. standard reads with a satisfactory degree of accuracy against solutions containing 0.05 to 0.015 mg. of phenol.

To determine the presence of conjugated phenols, 10 cc. of the 1:5 blood filtrate are put into a test-tube with 0.25 cc. of concentrated hydrochloric acid, and heated for 10 minutes in a boiling water bath. The solution is cooled and the added acid neutralized with sodium hydroxide. Acid and alkali are also added to the standard and to the unheated filtrate. The remainder of the determination is made as in the regular process.

Phenol added to blood is recovered quite satisfactorily by the nitroaniline method (Table I).

It seemed of particular interest to obtain comparative figures for free and conjugated phenols in blood by both the nitroaniline

TABLE I.

Showing Recovery of Phenol Added to Human Blood.

Except where otherwise indicated figures refer to mg. per 100 cc. of blood.

Phenol in blood.	Blood phenol + added phenol.	Theoretical phenol content.	Phenol recovered.
			per cent
1.17	1.97	1.79	110
	2.50	2.43	102
	3.02	2.99	101
1.23	1.75	1.84	95
1.56	2.93	2.81	104
	3.60	3.38	106
1.84	2.23	2.46	90
	2.50	2.75	90
	3.47	3.08	112
Average.....			101

and Folin-Denis reagents. Such figures are reported in Table II, where comparative figures by the new method and the Rakestraw procedure are reported.

It is interesting to note that while the two methods yield results not differing greatly, those by the nitroaniline method are very definitely lower. Thus the average free phenol of the twenty bloods done by both methods shows 1.28 mg. of phenol per 100 cc. of blood by the nitroaniline method, and 1.68 mg. per 100 cc. by the Rakestraw method.

In our former paper (1) we expressed the opinion that human blood is free from detectable quantities of conjugated phenols.

We reached this conclusion as a result of finding that the increased phenol following hydrolysis which we noted in some instances amounted to only a few tenths of a milligram per 100 cc. of blood, a difference well within the error of the method employed. Pelkan

TABLE II.

Showing Comparative Figures for Free and Conjugated Phenols in Human Blood by the New Method and by the Rakestraw Procedure.

All figures represent mg. per 100 cc. of blood.

No.	Nitroaniline method.		Rakestraw method.	
	Free phenols.	Total phenols.	Free phenols.	Total phenols.
1	1.3		1.4	1.8
2	1.2		1.8	2.3
3	1.3		1.3	1.6
4	1.1		1.6	
5	1.1		1.3	
6	1.0		1.5	1.7
7	1.4		1.8	1.7
8	1.0		1.5	1.9
9	1.2		1.7	1.7
10	1.5	1.6	1.7	1.9
11	1.1	1.1	1.4	1.7
12	1.2	1.8	1.6	1.9
13	2.0	1.8	2.0	
14	1.5	1.7	1.8	
15	1.4	1.5	2.1	
16	1.2	1.4	1.5	
17	1.3	1.3	2.0	2.0
18	1.0	1.2	1.6	
19	1.4		1.7	
20	1.3	1.5	1.8	
21	1.2	1.4		
22	1.5	1.8		
23	1.7	2.2		
24	1.4	1.7		
25	1.6	1.7		
26	1.8	2.0		

(2) criticized us very freely for reaching such a conclusion, stating among other things that our method was at fault since it included boiling down of the blood filtrates, during which process phenols were volatilized. Pelkan was, however, mistaken in this matter.

We had carefully tested our distillates from blood filtrates and had failed to detect any phenol whatever in such distillates. We therefore conclude that blood contains no detectable quantity of volatile phenols. By reporting his very limited number of figures in milligrams per liter of blood instead of according to the usual custom, Pelkan apparently emphasized the conjugated phenols which he found. As a matter of fact, Pelkan's figures closely approximated the results we had obtained, so that the chief difference lay in the interpretation and in the mode of reporting the figures.

From the results given in Table II, it would appear that a small amount of conjugated phenol probably exists in some bloods. Sixteen cases done by the nitroaniline method show an average total phenol of 1.6 mg. per 100 cc. of blood as against 1.4 mg. of free phenol per 100 cc. of blood. The average total phenol of eleven cases done by the Rakestraw method is 1.8 mg. per 100 cc. of blood and the free phenol is 1.6 mg. per 100 cc. of blood. In several of the bloods there is no increase whatever by the nitroaniline or the Rakestraw procedures following hydrolysis with acid. The application of the nitroaniline method to urine is being studied.

SUMMARY.

A method is described by which phenols can be determined directly on the Folin-Wu filtrate.

Figures for free phenols in the blood were obtained ranging from 1 to 2 mg. per 100 cc.

Conjugated phenols probably occur in small quantity in some bloods, but are not demonstrable in all bloods.

The figures obtained by this method on twenty bloods average 0.4 mg. per 100 cc. lower than the figures of the Rakestraw method on the same bloods.

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A NOTE ON THE GRAVIMETRIC MICROCHEMICAL TECHNIQUE.

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In the technique of gravimetric micro analysis as performed by Donau[1] and Pregl (2), one of the most important parts is the preparation and handling of the filter. In the following note we will show that, in the same way as in volumetric analysis, it is in many cases possible to avoid the use of the filter and with great simplicity to attain very accurate results. The gravimetric estimation can easily be made in a small glass tube, and the precipitate washed out by means of the centrifuge and a rubber-tipped capillary tube. The accuracy of the determination depends in many cases, even with the precipitates regarded as least soluble, to a great extent upon the quantity of solution from which the precipitation is made and upon the quantity of water used for the transferring and washing of the precipitate (Emich[2]). In the small tube described below, the precipitation and washing can be performed with quantities so small, that they are surpassed in delicacy only in the technique of Donau. Another advantage is that the hygroscopicity of the filter, which in the case of the filter used by Pregl prevents the reproduction of the weight of the filter closer than 0.005 mg., is avoided (Pregl (2)). With careful work the weight of the tube can be reproduced to 0.001 mg. The only disadvantage of washing the precipitate by means of centrifuge and capillary tube is that some precipitates (*i.e.*, calcium oxalate, ammonium phosphate molybdate) sometimes form a film on the surface of the liquid from which they are precipitated, which, if touched by the capillary tube, sink down

[1] See description of Donau's methods given by Emich (1).

[2] Emich (1) p. 285.

or adhere to the capillary tube. To avoid this, it is necessary to maintain such conditions during the precipitation as facilitate the production of a coarse, crystalline precipitate. Furthermore, the stirring up of the precipitate during the washing should not be done by shaking; the washing fluid should be injected into the sediment with the capillary tube so that the precipitate is stirred up. The loss caused through the clinging of precipitate particles to the capillary tube is very small. With work that is at all careful, it should be inappreciable. In cases where alcohol can be used for the washing of the precipitate, this source of error is eliminated.

Fig. 1.

The shape of the precipitation tubes is represented in Fig. 1. They may be of Pyrex glass or of quartz, diameter 12 to 15 mm., length 30 to 40 mm., capacity 2 to 3 cc., and with conical bottoms as shown in the figure. It is preferable to have tubes of various sizes.

Before weighing, the tube is rubbed off with wet flannel, then with dry chamois; but if after rubbing it is not put in the drying oven, the rubbing in dry condition should be avoided because the electrical charging caused by the rubbing prevents exact weighing. For weighing, the tube is placed upon the pan of the micro balance. For the details of the use of the microchemical balance, we refer to Pregl's description. The tube is handled with a small pincette, the arms of which have been inserted into small pieces of rubber tubing.

For making a precipitation in the small tube we have to follow exactly the quantitative conditions of chemical analysis, reduced proportionately to the quantity of fluid with which we are working. For instance, in the separation of potassium from sodium, and weighing the potassium as potassium perchlorate after the method of Schlossing-Wense, the results of which are presented in Table I, I proceeded according to the description in Treadwell's Analytical Chemistry (3), using $\frac{1}{40}$ of the amounts of the reagents as given by him. The reagents are introduced into the tube by means of a rubber tip, mounted pipette, graduated in 0.01 cc. For each washing of the precipitate by the centrifuge, 0.2 to 0.4 cc. of the washing fluid is required. As

I mentioned above, the washing fluid is to be injected with a capillary tube in such a way as to stir up the precipitate; then, by carefully tilting the tube we wash off the walls with the washing fluid. For precipitates which do not strongly retain impurities, four washings should be sufficient.

In a large series of determinations, the four washings of the precipitate require only 4 or 5 minutes to each tube, which is not longer than the time required for the transferring and filtration of precipitates.

TABLE I.

Weight of K used for the analysis.	NaCl present in the solution.	K found.	Error.
mg.	*mg.*		*per cent*
1.680	9.612	1.673	−0.4
1.540	8.811	1.535	−0.38
1.639	9.375	1.633	−0.35
1.533	8.769	1.544	+0.72
0.761	4.353	0.762	+0.14
0.724	4.143	0.727	+0.5

TABLE II.

Weight of Ca used for the analysis.	Weight of the Ca oxalate precipitate.	Ca found.
mg.	*mg.*	*mg.*
0.744	2.717	0.744
0.7175	2.619	0.7175
0.2858	1.042	0.2854
0.2675	0.977	0.2677

I did not try to heat the precipitates in quartz tubes. Even with precipitates where heating seems almost unavoidable, such as barium sulfate and magnesium ammonium phosphate, as was recently shown, we can get very good results under appropriate conditions without heating.

In Tables I and II I give, as illustrations, the results of determinations of calcium as calcium oxalate, and of potassium as potassium perchlorate in the presence of three times as much sodium. For the calcium I followed the description given by Lockemann (4), for the potassium that of Treadwell, as men-

tioned above. The calcium solution was made by dissolving the purest calcium carbonate in hydrochloric acid, the potassium solution by dissolving the purest potassium chloride and sodium chloride. In the last three determinations, the potassium was not precipitated twice, but only once, with the perchloric acid.

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FURTHER STUDIES ON THE DETERMINATION OF CALCIUM, MAGNESIUM, AND PHOSPHORUS IN ANIMAL SUBSTANCES.

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In 1919 I published a method of estimating very small quantities of calcium, magnesium, and phosphorus in animal substances (1). The method followed the system in micro chemistry which is best represented by Emich, Donau, and Pregl; *i.e.*, to proceed according to the methods tried and used in ordinary chemical analysis, and to adapt the technique to the handling of very small quantities. The technique used, which is an adaptation of the microchemical technique already in use to special purposes, differs quite considerably from the technique generally used in chemical work, and also differs markedly from the microchemical methods used in biochemical work. Probably that is one of the reasons why the method I described, which gives very accurate results and can be performed in a relatively short time, has found little general consideration. As I believe that this technique not only gives good service in the cases studied by me, but can be applied to the solution of other problems of microchemical analysis, I consider it appropriate to describe my further experience with it. In my publication of 1919, I described only a procedure and results with volumetric methods. In a subsequent paper I will demonstrate that, with the same simple technique, very exact results can be obtained with gravimetric methods also.

Since the publication of my former paper there have appeared several publications describing methods of determining small quantities of calcium by titration with potassium permanganate, and some of these are extensively used. However, as my method is quite different, and was not influenced by these, it seems to me

unnecessary to go into any detailed account of the literature of the subject.

Determination of Ca.

In every chemical analysis the problem is divided into two parts. The first is the real method of estimation; the second, to bring the substance under analysis into a form appropriate to the determination. For the determination of calcium, I used the precipitation with oxalic acid and the titration of the precipitate with potassium permanganate. I have shown that the solubility of the calcium oxalate precipitation is large enough in the liquid from which it is precipitated, so that the error can be kept under 0.001 mg. (which is 1 per cent of the calcium present in 1 cc. of human plasma), only in case the volume by the precipitation remains under 1 cc., and the solubility of the precipitate in distilled water so high, that washing with only 0.7 to 1.2 cc. of water, the smallest quantity with which I succeeded in washing out the precipitate, results in a loss of 0.002 to 0.004 mg. of calcium. This difficulty was avoided by precipitating the calcium in a small quartz tube and, instead of washing out the precipitate, measuring the quantity of oxalate added, and weighing the tube with the whole contents and again weighing after pipetting off the liquid above the centrifuged precipitate. In this way the amount of oxalate remaining with the precipitate can be calculated. As the weight of the tube has to be known only to an accuracy of 1 mg., and as this is approximately known, the weighing does not take excessively long and the time needed for the weighing is less than that required to wash out the precipitate. The accuracy of this method, with a few tenths of a milligram of calcium, is equal to that of any macroanalytical method and is practically limited only by the amount of potassium permanganate solution which can be recognized in the titration, which amount is, in the small tubes used, 0.001 to 0.002 cc. of a 0.01 N solution. The very small amounts of calcium, as I have formerly pointed out, have to be determined in a small, thin tube, otherwise the result comes out 0.002 to 0.003 mg. higher. But even when small tubes are used for very small quantities, we always get a little higher results.

In Table I it may be seen that amounts down to 0.04 to 0.02 mg. can be determined with 1 per cent exactitude. At the same time this table shows that the presence of 2.5 per cent ammonium acetate does not influence the results of the determinations, but with 10 per cent the results are appreciably lower.

I have shown in my previous paper that the exactitude of the method is not the result of compensating errors, but that the mother solution of the precipitate really does not contain more calcium than is lost by the determination.

TABLE I.

Weight of Ca used for the analysis.	Ammonium acetate present in the solution.	Ca found.	Error.
mg.	*per cent*	*mg.*	*mg.*
0.1938	10	0.1916	−0.0022
0.1980	10	0.1970	−0.0010
0.1609	2.5	0.1616	+0.0007
0.1855	2.5	0.1854	−0.0001
0.2048	None.	0.2046	−0.0002
0.1888	"	0.1882	−0.0006
0.0288	"	0.0290	+0.0002
0.0315	"	0.0316	+0.0001

In the last two determinations the volume from which the Ca was precipitated was 0.32 cc. with the others approximately 1 cc.

Although the precipitation and titration of calcium are simple processes and give satisfactory results, the method of preparing the material for the precipitation of the calcium as described by me has caused considerable difficulty to some who tried to use it. The difficulty lies mainly in this, that if the ash is heated too much, or if by the driving off of the ammonium salts too high temperature is used, the calcium cannot be brought into solution with the amount of hydrochloric acid which is used in microchemical analysis. To avoid this difficulty and at the same time make the execution of the analysis easier, there was introduced the following modifications. The ash, after burning, is fused with a small amount of sodium carbonate, which renders it easily soluble in hydrochloric acid. But, as by this procedure some ferrous salts are produced, I add to the solution of the ash a small amount of hydrogen peroxide. The precipitation of phosphorus is made in

small volume, so that the evaporation of the solution and the driving off of ammonium acetate can be avoided. Formerly I had not in my possession any specimen of acetic acid which did not reduce the potassium permanganate solution to some degree. Later I obtained several specimens which, in the quantities present during the precipitation of the phosphorus, do not reduce any potassium permanganate solution. On the other hand, the presence of the ammonium acetate in greater concentration increases the solubility of calcium, and we have to avoid the presence of more than 2 to 2.5 per cent. These changes in the procedure, if the quantitative requirements later described are followed exactly, do not alter the accuracy of the method. However, if the highest accuracy is required with less than 0.1 mg. of calcium, the original method seems to be preferable. Finally, instead of quartz tubes, Pyrex glass tubes may be used which do not contain appreciable amounts of lime salts.

In the description of the method, I start with 2 cc. of blood, or 1 cc. of plasma. By using these quantities the parallel determinations will mostly agree within 1 per cent. Later I add the quantitative requirement by using 0.5 to 0.6 cc. of blood, or 0.3 to 0.4 cc. of plasma. By using smaller quantities than 2 cc. of blood or 1 cc. of plasma, if the highest accuracy is required, after the precipitation of phosphorus and iron, the ammonium acetate has to be driven off as mentioned above.

Apparatus Required.

Quartz or Pyrex tubes, 70 mm. long, 8 to 9 mm. inner diameter, with mark at 1.2 cc.

Quartz or Pyrex tubes, 60 mm. long, 7 to 8 mm. inner diameter.
" " " " 40 " " 5 mm. inner diameter.

The two smaller sized tubes should have conical bottoms, not round ones.

Glass capillaries of Pyrex glass, of about 2 to 2.5 cc. content, mounted with rubber tips, as used in the serological methods of Wright.

Burettes graduated in 0.01 cc., ending in fine capillary tubes.

Sodium carbonate, 5 per cent solution.
Hydrochloric acid, 25 " " "
Hydrochloric acid, 1 " " "
Ammonium acetate, about 50 to 60 per cent solution.
Ferric chloride, 5 per cent solution.
Hydrogen peroxide, 3 " " "
Ammonia, 10 " " "

Ammonium chloride, 40 per cent solution.
Oxalic acid, 0.1 N.
Potassium permanganate, 0.01 N.
Sulfuric acid, 20 per cent solution.

For the distillation of water a Pyrex cooler was used. The hydrochloric acid and the ammonia solutions should be made by introducing the gaseous reagent with a Pyrex tube into the distilled water, the ammonium acetate by neutralizing acetic acid with ammonia. In all cases, the calcium content of the reagents should be determined. I prefer to evaporate twenty times the required amounts of the reagents together, and to determine the calcium content of the whole. The reagents are kept in small Pyrex flasks and are taken out of them with capillary tubes or pipettes.

Procedure.

2 to 2.5 cc. of blood, or 1 to 1.5 cc. of plasma, are weighed on the analytical balance in a small platinum crucible of 10 cc. capacity, then dried on the water bath, and after complete drying, with covers on, dried at a temperature slowly elevated from 120° to 160°C. in the drying oven. After this procedure, the crucible is placed on a triangle and, beginning by heating the side walls, the crucible can be heated without danger of jumping. When the melted contents again solidify and no more fumes come out, the crucible is placed in a porcelain crucible with asbestos on the bottom, or in an oven, and the contents are ignited at as low temperature as possible. Sometimes it is necessary to stir the ash with a platinum wire. When the ashing is complete, 0.1 cc. of 5 per cent sodium carbonate is added to the crucible, and with this, by carefully tilting the crucible, the side walls are washed off as high as the ash reaches. The crucible is again dried on the water bath, and with cover on is dried in the drying oven, the temperature being slowly elevated from 100° to 160–180°C. After this the crucible is placed on a triangle, and beginning the heating with the side walls, the small content of the crucible is melted. Then 3 or 4 drops of distilled water are put into the crucible. 3 drops of concentrated hydrochloric acid are put on the cover to dissolve the particles which accidentally jumped there. The hydrochloric

acid is dropped on the cover, then with a capillary tube mounted with a rubber tip, by alternately taking up the hydrochloric acid into the tube and letting it out again on the cover, we wash off all the surface which may have any of the particles. The hydrochloric acid is then dropped into the distilled water in the crucible. The cover is again washed with a few drops of distilled water. After this the crucible is placed over a small hole in the water bath and, washing the side walls off with the capillary tube, the contents are dissolved and then evaporated to dryness. The contents are then taken off with 1 or 2 drops of 1 per cent hydrochloric acid and a few drops of distilled water by heating the crucible on the water bath, and transferred to a Pyrex tube with the capillary pipette. The crucible is washed four or five times with 3 or 4 drops of distilled water with the aid of the capillary tube, each time washing off the whole inner side wall of the crucible. The Pyrex tube is filled with distilled water, almost to the 1.2 cc. mark, well mixed, then 0.05 to 0.08 cc. of ammonium acetate solution is added, and the contents are again well mixed. If the mixing presents any difficulty, a capillary tube may be used for this purpose, and afterwards washed with a few drops of water. Then enough ferric chloride solution is added to render the solution frankly orange colored, and also 0.04 cc. of a 1:100 dilution of 3 per cent hydrogen peroxide solution. After mixing, the tube is covered with tin-foil, centrifugalized for a few seconds to bring down the fluid hanging on the wall, and is immersed almost to the top in the boiling water bath. After 1 to 2 minutes, the tube is taken out and placed in water to such a depth, that only the part containing the solution is immersed in the water. By proceeding in this way, we succeed in preventing any condensation on the wall of the tube. This is necessary, because, if we shake the tube after the precipitation of iron, part of the precipitate becomes so finely distributed that we cannot obtain an iron-free solution by centrifugalization. After cooling the tube, it is covered with tin-foil, centrifugalized, weighed on the analytical balance, the clear solution pipetted off into a Pyrex tube of 60 mm., and the tube again weighed with the iron precipitate. The pipette is washed out twice with a drop of water, drawing the drop each time as high as the ash solution formerly was, and letting it out into the smaller Pyrex tube. To the solution in the Pyrex tube is added 1 drop

of 10 per cent ammonia, and 0.05 cc. of 40 per cent ammonium chloride, the contents are mixed, then put into the water bath and a few minutes later, while still in the water bath, 0.2 to 0.25 cc. of ammonium oxalate solution is added from a burette. The amount of oxalate added is to be noted. After 1 or 2 minutes the tube is taken from the water bath, and is left standing overnight. Sometimes it forms a small iron precipitate in the tubes. This is of no significance and does not influence the results. The next day, by carefully tilting the tubes, the walls are washed off by the contents. The tube is centrifugalized, weighed, and the supernatant fluid pipetted off into another tube for the determination of magnesium (pipette washed out as described above). With some practice the pipetting with the rubber-tipped capillary pipette can safely be done so that only a few centigrams of the fluid remains over the sediment. The pipetting off of the fluid is sometimes rendered difficult by particles of the precipitate floating on the surface and adhering to the capillary tube. The error caused by this can be avoided by discharging a small drop from the capillary tube after finishing the drawing off of the fluid, and touching the wall of the tube with the droplet.

The titration is made with a 0.01 cc. graduated burette ending in a fine capillary tube, with carefully ground stop-cock which can be used without grease. To the tube is now added 0.1 cc. of 20 per cent sulfuric acid, and it is held for a short time in a water bath at 60°C. Then the potassium permanganate solution is added, at first faster, and when the end of the titration is approaching, by carefully introducing only 0.01 cc. at a time. How much potassium permanganate solution is required is in most cases approximately known, and from this we must decide when to begin with 0.01 cc. During the titration the tube is heated to 60°C. several times. At the end of the titration we determine how much potassium permanganate solution has been added in excess of the requirement, by adding potassium permanganate solution to an equal amount of distilled water in a similar tube, until the same coloration is reached. The titration as described takes more time than a procedure with drops, but the results, as I have shown in my previous paper, and as illustrated in Table I, are of very high accuracy. It seems to me necessary to use not more than 0.01 cc. of permanganate at the end of the titration, otherwise the

colorimetric determination of the permanganate added in excess is not possible.

If we use 0.5 to 0.7 cc. of blood, or 0.3 to 0.4 cc. of plasma, a Pyrex tube of 60 mm. is used for the precipitation of phosphorus. The ash is dissolved in only 0.6 cc. of distilled water, and 0.04 cc. of ammonium acetate and 0.02 cc. of 1:100 dilution of 3 per cent hydrogen peroxide are added. After proceeding as previously described, the clear solution after the iron precipitation is brought over to the Pyrex tube of 40 mm., and after the addition of a small drop of ammonia and 0.03 cc. of 40 per cent ammonium chloride, it is precipitated with 0.1 cc. of ammonium oxalate.

I considered it necessary to make the preceding description very detailed, as the technique differs from the usual technique, and all these details are essential in order to obtain accurate results. Most of the operations can either be performed in a very short time, or do not require watching, as, for instance, the ashing. The two weighings and the pipetting off of the tubes, if done in a series, take only 5 minutes to each tube, a time not considerably more than would be required for any washing of the precipitate. The procedure can be simplified by reading the volume of the solution in a graduated tube instead of weighing the tube, but even by using a little more blood and larger volumes, as described above, 2 to 3 per cent error can occur from errors in the reading. According to my knowledge, there is no scale with which a determination of weight of 1 to 10 gm. can be made within 1 mg. accuracy, as easily as with the spring scale used by I. Bang. I have constructed a scale on the aerometer principle, of which I hope to be able to give an account in the near future.

Determination of Mg and P.

For the determination of magnesium there were made no changes in the technique already described, except that I find that in many cases the precipitation of ammonium phosphate molybdate as used by Lorenz[1] and Pregl (3) is preferable to the method already described, which follows the method of Woy. For this I use the reagents as described in Pregl's book.

[1] For a description of the von Lorenz method see Neubauer and Lücker (2).

To the Pyrex tube containing the supernatant fluid of the calcium precipitate is added 0.2 cc. of a secondary sodium phosphate or secondary potassium phosphate solution which contains 2 mg. of phosphorus to each cubic centimeter. The tube after mixing is heated in the water bath, then 1/10 volume of 20 per cent ammonia is added drop by drop, and mixed. The walls of the tube (after the procedure of Briggs (4)), are rubbed with a thin glass rod without rubber tip. The tube is left standing overnight, then, after the walls of the tube are washed off by the contents, the tube is centrifugalized, weighed, the contents are pipetted off, and again weighed. To the contents of the tube are added 0.10 cc. of 24 per cent nitric acid and distilled water up to 0.5 cc., mixed, the walls washed with the contents, and heated in a water bath in such a way that only the part of the tube containing the fluid is immersed. By again carefully tilting the tube, the walls are made wet, and then 0.5 cc. of the molybdate reagent is injected directly into the fluid contents of the tube with a thin pipette or capillary tube, great care being taken to prevent any of the molybdate reagent from reaching a part of the tube which is not wet. The contents of the tube are mixed and the tube is again placed for 40 to 50 seconds in the water bath and immersed to the depth of the contents. It is then placed in a wet chamber (a beaker containing some distilled water and covered with a watch-glass) for a few hours, then washed twice by centrifugalizing with 2 per cent ammonium nitrate, and twice with 1 per cent potassium nitrate. After washing, the sediment is dissolved with 0.1 N sodium hydroxide, phenolphthalein is added to a deep red color, and titrated back with 0.05 N or N/30 hydrochloric acid. 1 cc. of 0.1 N sodium hydroxide corresponds to 0.1262 mg. of phosphorus. This procedure with small amounts of phosphorus seems to be more advantageous than the precipitation with 3 per cent ammonium molybdate according to Woy, because, down to 0.01 mg., the amount of sodium hydroxide is proportional to the phosphorus present, which is not the case when using 3 per cent ammonium molybdate for the precipitation.

In Table II are presented a series of determinations of phosphorus after this method.

It seems to me that with very small quantities, 0.05 to 0.01 mg., this volumetric method is more accurate than the gravimetric. With larger amounts the gravimetric seems to be more accurate. The washing of the molybdate precipitate in a large series of determinations takes an average of 4 to 5 minutes to a tube. It seems to me necessary, from time to time, to make a determination with a solution of known phosphorus content, to control the reliability of the reagents.

TABLE II.

Weight of P used for the analysis.	0.1 N NaOH required for the titration of 0.1 mg. P.
mg.	*cc.*
0.2030	0.789
0.1916	0.795
0.1164	0.792
0.1141	0.785
0.0919	0.792
0.0251	0.795
0.0241	0.794
0.0239	0.796
0.0145	0.787
0.0124	0.792
Average.....	0.7922

In Table III are presented the results of determinations of calcium and magnesium in a specimen of sheep's blood to which was added 1:10, 5 per cent phenol solution. The determinations made with the smaller quantities are on the average 2.5 per cent higher than those made with larger quantities. However, this difference does not correspond by the titration to more than 0.003 cc. of potassium permanganate solution. Why the smaller amounts give slightly higher results, I was unable to determine. In using smaller amounts for determination and proceeding as above described, we have to introduce a correction. For the magnesium no correction is needed.

Calculation of Results.

A concrete example will show how the calculations of the results are made. In the first specimens of Table III the quantities were:

2.740 gm., weight of blood.
1.684 " " " fluid from which the phosphorus was precipitated.
1.417 " " " " separated from the iron phosphate precipitate and used for the precipitation of calcium.
1.832 gm., weight of fluid separated from the calcium precipitate.
0.026 " " " " remaining with " " "
0.35 cc. of N/15 oxalic acid was used for the precipitation of calcium corresponding to 2.333 cc. of 0.01 N potassium permanganate solution.
0.642 cc. of 0.01 N permanganate was used for the titration of calcium oxalate, and of the fluid remaining with it.

TABLE III.

Amount of blood used for the analysis.	Ca found in 1 cc. blood.	Mg found in 1 cc. blood.
gm.	*mg.*	*mg.*
2.740	0.0530	0.0321
2.598	0.0532	0.0324
2.477	0.0532	0.0324
2.402	0.0528	Not determined.
Average.....	0.05305	
0.603	0.0548	Not determined.
0.652	0.0536	" "
0.559	0.0544	0.0323
0.672	0.0548	0.0325
Average.....	0.0544	

To obtain the amount of permanganate used for the titration of the calcium oxalate precipitate we have to deduct from 0.642 cc. the amount of permanganate corresponding to the oxalate contained in the fluid remaining with the precipitate.
This amount is

$$\frac{2.333 - 0.624}{1.832} \times 0.026 = 0.024$$

The amount of permanganate corresponding to the whole amount of oxalate used for the precipitation of calcium minus the amount of permanganate found by the titration, represents the amount of oxalate pipetted off from the precipitate. Dividing this by the amount of fluid pipetted off from the precipitate, and multiplying it by the amount of fluid remaining with the precipitate, we obtain the amount of permanganate corresponding to the oxalate remaining with the precipitate.

0.642−0.024 = 0.618 multiplied by 0.2 gives us the quantity of calcium present (0.1236 mg.) in that part of the ash solution which was used for the precipitation of calcium. To obtain the calcium content of the whole ash solution we have to divide this by the weight of the fluid pipetted off from the iron phosphate precipitate, and multiply by the weight of the whole fluid from which the phosphorus was precipitated,

$$\frac{0.1236 \times 1.684}{1.417} = 0.1469$$

From this we have to deduct the calcium contained in the reagents (0.0016 mg.), and divide it by the amount of blood used for the determination. In this way we obtain the calcium content of 1 cc. of blood,

$$\frac{0.1469 - 0.0016}{2.740} = 0.0530 \text{ mg.}$$

For the calculation of magnesium from the amount of phosphorus found by the titration, we have to deduct the amount of phosphorus contained in the small quantity of fluid remaining with the magnesium ammonium phosphate precipitate, calculated in the same way as we have above calculated the amount of oxalate remaining with the oxalate precipitate. 0.784 mg. of magnesium corresponds to each mg. of phosphorus. To the amount of magnesium present in the precipitate we have to add the amount of magnesium contained in the small amount of fluid remaining with the calcium oxalate precipitate, and the amount remaining with the iron phosphate precipitate. Both of these are calculated as we calculated the calcium remaining with the iron phosphate precipitate.

In the calculation of the amount of calcium and magnesium remaining with the iron phosphate precipitate, I do not take into consideration the weight and the volume of the iron phosphate precipitate. Using 2 cc. of blood, the weight of the iron phosphate precipitate amounts approximately to 3 mg. I formerly found, empirically, that from a mixture of salts nearly corresponding to the salts of the blood ash, omitting the weight of the iron precipitate from the calculation, I obtained exact results. The iron precipitate, on the other hand, does not absorb appreciable amounts of calcium, as was recently confirmed by Hirth and Klotz (5). The uncertainty introduced by the above method of calculation is very small, and it can be avoided either by using larger volumes for the precipitation of phosphorus, which necessitates the evaporation of the fluid after the precipitation and expulsion of ammonium salts, or by washing out the iron phosphate precipitate. The weight of calcium oxalate precipitate to 0.1 mg. of calcium is approximately 0.36 mg., and this does not cause appreciable error.

For the determination of the calcium content of the blood, the most accurate method seems to me, as I formerly described it, to use the plasma that is obtained by centrifugalization of the cooled blood. By this procedure we can, in the easiest way, obtain 2 to 3 cc. of plasma. In the estimation of calcium, the determinations being made in a specimen of the blood, the plasma, and the red cell residue, we obtain, with three determinations, parallel determinations for the blood and for the plasma. Unfortunately, taking into consideration the small amount of magnesium present in the plasma, this method is not satisfactory for parallel determinations of plasma magnesium.

SUMMARY.

An account is given above of some modifications of the method of determination of calcium, magnesium, and phosphorus in animal substances described by me in a previous publication. Both with calcium and magnesium, the parallel determinations mostly agree as closely as 0.001 mg.

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A NOTE ON THE RÔLE OF THE LIVER IN PARATHYROID TETANY.

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As a result of recent work on the syndrome known as parathyroid tetany, there are today two current views as to the origin of the condition that have considerable evidence in their favor. The first, that the tetany is due to lowering of the calcium content of the blood, is supported chiefly by evidence from blood analyses. The second, that the condition is due to an intoxication arising from the gastrointestinal tract, is upheld for the most part by indirect evidence. The work of Dragstedt (1, 2) and of Luckhardt (3–7) and their coworkers give strong support to the latter view, although their conclusions have been denied by the supporters of the calcium theory, because much of their work may be interpreted on either theory.

The work done heretofore has thrown little light on the mechanism by which the parathyroids prevent tetany. In an effort to test the validity of the two current theories experimental work was begun on the basis of the following reasoning.

If parathyroid tetany is due to an intoxication arising from the gut, and if the condition is due to the passage into the circulation of bodies that literally swamp the liver and other organs, due either to an increased absorption of such substances or to an inability of the liver to detoxify them, or both; then in an animal whose portal circulation has been so altered that the blood from the gastrointestinal tract reaches the liver in extreme dilution as compared to normal, there should be a corresponding increase in severity of the tetany symptoms. Furthermore, if the methods of control of tetany depend upon a neutralization or destruction of circulating toxins, by augmenting hepatic de-

toxification, such an animal should be very difficult to keep under control by diet, calcium rations, or diuresis. But if the primary cause of the tetany condition is a lowering of the blood calcium, then the circulatory changes in an Eck fistula dog should have little or no effect on the symptoms following parathyroidectomy.

That the depression of the detoxifying power of the liver is a factor in tetany has been suggested by many authors. Animals that have extensive liver lesions produced experimentally by phosphorus or chloroform poisoning will exhibit toxic symptoms after excessive meat diet. And it was furthermore shown by Dragstedt (2) that a parathyroidectomized animal controlled on an aciduric diet would pass into a tetany condition following poisoning by phosphorus within 48 hours.

In this work dogs were thyroparathyroidectomized at varying periods after the establishment of an Eck fistula. In this communication a summary of the results obtained on the first series of six dogs will be presented; the complete protocols will be included in a later publication.

Dogs were thyroparathyroidectomized approximately 6, 3, and 1 month, and 2 weeks after the establishment of an Eck fistula. In every case the animals were closely watched for symptoms of meat intoxication previous to the parathyroidectomy, and none of the animals used in this series showed such symptoms.

In such dogs, following parathyroidectomy, there is a delay in appearance and a diminution in the severity of the symptoms as compared to an ordinary parathyroidectomized animal. The first symptoms to appear are those of meat intoxication; the animal appears to suffer from hallucinations, has spasmodic jerkings, but unlike tetany, there is no hyperpnea accompanying these seizures. This condition becomes progressively worse if no methods of treatment are employed, and finally typical attacks of tetany ensue. In one animal no symptoms other than a mild meat intoxication ever appeared, even on a forced meat diet. In these animals the time between the operation and the appearance of the first symptoms of intoxication, on a straight meat diet, varied from 6 to 17 days.

In every case all symptoms (meat intoxication and tetany) can be abolished with the greatest ease by the administration of

calcium salts *per os*. The amounts required, however, are much smaller than the minimum dosage worked out by Luckhardt (4), who found that at least 1.5 gm. per kilo of body weight per day are necessary to control an animal. Our dogs were kept in excellent condition with about one-third to one-fifth that amount. Moreover, the rapidity of recovery from an attack is much greater than in simple parathyroidectomized dogs, and such animals even when in acute tetany rarely vomit a solution given by stomach tube.

Without exception it was found that Eck fistula-thyroparathyroidectomized dogs do not pass into a condition in which the calcium treatment may be suspended. After the first appearance of the tetany symptoms these dogs were placed on a daily diet of 0.5 pound of meat and 0.5 pound of bread, with suitable amounts of calcium lactate or other calcium salts. When, after 5 or 6 weeks, they were placed on the laboratory stock diet, they passed into a tetany condition. There is, in other words, no compensatory adjustment after 30 to 40 days of control as described by Luckhardt and by Dragstedt. As far as we can judge at present, there is no progressive increase in the severity of the symptoms; there is only a persistence.

The appearance of bilateral, presenile cataract in such animals, previously described by Luckhardt and Blumenstock (5) as occurring in parathyroidectomized dogs, is a constant factor.

Such animals, in spite of the ease with which they may be controlled by small amounts of calcium salts, cannot be maintained free from tetany on a milk diet alone. One of these dogs after being on a straight milk diet for 12 days went into typical intoxication, followed by an epileptiform seizure which passed into unmistakable tetany. This has been repeatedly confirmed.

We hesitate to present conclusions or final interpretations on the basis of only six dogs. But the following points may be worthy of consideration.

1. The rôle of the liver as the detoxifying agent in tetany seems to be questioned. It has been demonstrated that Eck fistula dogs are more resistant to chloroform poisoning (8), also to phosphorus poisoning (9). Our results indicate that substances absorbed from the gut are not the direct toxic agents as such, but they may cause tetany after being modified by some

tissues, most probably the liver; the toxic substances absorbed from the gut may cause hepatic destruction which then gives rise to the tetanic condition. The latter interpretation would explain why an increase in permeability of the gut will promote tetany as recently shown by Luckhardt and Compere (7). It would also explain the difficulty of controlling by dietary measures parathyroidectomized animals who are pregnant or are in "heat," for it is possible in such animals that substances that cause hepatic injury enter the circulation from sources other than the gut.

2. It is difficult to explain our present results on the basis of the calcium deficiency theory. The ease of control, and the change in the syndrome in these dogs is itself an argument against such a theory; for if the parathyroids control the fixation of calcium in the tissues, there should be little or no change in the syndrome in an Eck fistula animal. It was an early view of Luckhardt that the *calcium deficiency in parathyroidectomized animals might well be an effect of the tetany condition rather than its cause* (6). Our work supports that view.

3. Salvesen, a supporter of the calcium deficiency theory, claims to have controlled parathyroidectomized dogs by a milk diet, and claims the control is due to the calcium content of the milk (10). If this is true, our animals who may be controlled with but a fraction of the dosage of calcium necessary for normal parathyroidectomized dogs, should never have gone into an intoxication and tetany on a milk diet.

4. The possibility of a hyperplasia of accessory parathyroid tissue has been brought up by Shapiro and Jaffé (11) as an explanation for the "cure" of parathyroidectomized dogs after 30 to 40 days of treatment with diet, diuresis, or calcium feeding by the methods of Luckhardt and of Dragstedt. These latter authors considered this possibility and dismissed it as not accounting for all the facts. The invariable persistence of symptoms in our dogs is evidence in itself that if there were accessory parathyroids in these dogs, they were not present in sufficient amount to prevent persistent deficiency symptoms.

5. The failure to obtain a cure, or rather a restoration, to nearly normal conditions after 30 to 40 days of calcium control in our parathyroidectomized-Eck fistula dogs may be due to the

progressive degeneration of the liver that is a constant occurrence in Eck fistula animals.

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A CONTRIBUTION TO THE CHEMISTRY OF GRAPE PIGMENTS.

II. CONCERNING THE ANTHOCYANS IN CLINTON GRAPES.

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INTRODUCTION.

It has been shown in an earlier paper from this laboratory[1] that the pigments occurring in two varieties of American grapes, *viz.* Norton (*Vitis æstivalis, labrusca*) and Concord (*Vitis labrusca*), were identical and that they had the same chemical composition as the monoglucoside oenin isolated from *Vitis vinifera* by Willstätter and Zollinger.[2] In continuation of the investigation of grape pigments we have examined the coloring matter that occurs in Clinton grapes. Clinton[3] is said to be the first cultivated variety of *Vitis riparia,* but it is believed that it also contains some blood of *Vitis labrusca.*

The pigment occurring in Clinton grapes was found to be identical with the anthocyanins isolated from Norton and Concord grapes and it was apparently identical with the anthocyanin isolated from *Vitis riparia* by Willstätter and Zollinger.[4] The anthocyanin consists principally of a monoglucoside. It was isolated as the picrate and the latter was converted into the chloride. The anthocyanin chloride could not be obtained in definitely crystalline form. It was precipitated by ether from its solution in methyl alcohol as amorphous flakes and it separated

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[2] Willstätter, R., and Zollinger, E. H., *Ann. Chem.*, 1915, cdviii, 83.
[3] Hedrick, U. P., Grapes of New York, *N. Y. Agric. Exp. Station,* 1908.
[4] Willstätter, R., and Zollinger, E. H., *Ann. Chem.*, 1916, cdxii, 195.

slowly from a solution in a mixture of methyl and ethyl alcohol and dilute hydrochloric acid in the form of small globular particles. The anthocyanin picrate crystallized in beautiful bright red prisms and this salt was prepared and analyzed. It corresponds to the formula $C_{23}H_{24}O_{12} \cdot C_6H_2(NO_2)_3OH$.

The anthocyanin from Clinton grapes has the same percentage composition as oenin, derived from *Vitis vinifera*, but it differs from this substance in that it does not form a crystalline chloride and also in that it gives in alcoholic solution an intense purple coloration with ferric chloride. The color reaction with ferric chloride is identical with that reported in our first paper[1] and similar to that described by Willstätter and Zollinger[4] for the pigment isolated from *Vitis riparia*.

The absorption spectrum is practically identical with that described in our first paper.[1] It consists of one broad band extending from the yellow into the blue.

The anthocyanin chloride is easily hydrolyzed when boiled with 20 per cent hydrochloric acid, forming 1 molecule each of glucose and anthocyanidin chloride, the latter crystallizing from the hot solution in beautiful prisms.

The anthocyanidin chloride has the same percentage composition as oenidin chloride, but it differs from this substance in that it contains a lower percentage of methoxyl. The amount of silver iodide obtained in the Zeisel determination corresponded more nearly to one methoxyl, but the values obtained were always high. It is probable that the substance is a mixture of mono- and dimethyl ethers of delphinidin. The reaction mixture from the Zeisel determination deposited, after the solution had cooled, small red prismatic crystals of delphinidin iodide. The delphinidin iodide was transformed into the chloride by the method described by Willstätter and Zollinger[2] and this salt was analyzed.

We were able to isolate and identify phloroglucin as one of the decomposition products formed on fusing anthocyanidin chloride with potassium hydroxide, but we were unable to isolate any gallic acid. A derivative of the gallic acid part of the molecule was obtained by another method. The anthocyanidin chloride was boiled with acetic anhydride and the resulting acetyl derivative was oxidized with neutral permanganate. The reaction mixture gave on extraction with ether an acetylated methyl ether of gallic

acid that contained nearly the same proportion of methoxyl as the anthocyanidin chloride itself. The amount of the gallic acid derivative was too small to permit of its identification, but the results indicate that the methyl groups are attached to the gallic acid part of the molecule and not to the phloroglucin.

EXPERIMENTAL PART.

The skins were separated from the pulp by hand and pressed in a hydraulic press. The pressed skins, 13 kilos, were digested for about 20 hours in 13 liters of 0.5 per cent hydrochloric acid and they were then rubbed to a pulp in a mortar. The liquid was expressed in a hydraulic press and the residue was stirred up with about one-half of its weight of 0.5 per cent hydrochloric acid and again pressed. The solution thus obtained was filtered through a layer of paper pulp yielding an intensely deep red and perfectly clear filtrate that measured 22 liters. It was warmed on the water bath to about 45° and 225 gm. of finely powdered picric acid were added and the mixture was stirred until the picric acid was dissolved. The picrate separated slowly, after the solution had cooled, in bright red needle-shaped crystals. The crystals were collected on a Büchner funnel and dried in a vacuum desiccator over sulfuric acid and potassium hydroxide. The crude picrate obtained in this way weighed 64 gm.

The picrate was recrystallized from water and analyzed.

Analyses. 0.1912 gm. dry substance: 0.0689 gm. H_2O and 0.3373 gm. CO_2.

Calculated anthocyanin picrate, $C_{29}H_{27}O_{19}N_3$ (721). C 48.26, H 3.74 per cent.

Found. C 48.11, H 4.03 per cent.

The product lost 5.49 per cent of its weight on drying at 105° in a vacuum over phosphorus pentoxide, corresponding to slightly more than 2 molecules of water of crystallization.

Calculated for $C_{29}H_{27}O_{19}N_3 + 2\ H_2O$ (757). H_2O 4.75 per cent.

Conversion of Anthocyanin Picrate to the Chloride.

The picrate was converted to the chloride according to the method of Willstätter and Zollinger. The crude picrate was dissolved in 575 cc. of methyl alcohol, and 100 cc. of 20 per cent

hydrochloric acid in methyl alcohol were added. The anthocyanin chloride was precipitated by the addition of 5,200 cc. of ether. It was filtered and washed with ether until free from picric acid. After drying in a vacuum desiccator it weighed 25 gm.

The anthocyanin chloride prepared in this way is a brownish red powder, easily soluble in water giving a brownish red solution which becomes bright red on addition of acid. The aqueous solution of the pigment gives with a dilute solution of sodium acetate a purple color; carbonates produce a deep blue color, whereas sodium hydroxide gives a bluish green color which soon changes to brownish yellow. Alkali destroys the pigment since the red color cannot be restored by the addition of acid. The pigment is completely precipitated by lead acetate forming a deep blue amorphous precipitate. An aqueous solution of the pigment gives with 1 drop of very dilute ferric chloride a purple coloration which passes rapidly through wine-red to brown. In alcoholic solution a fairly permanent violet coloration is produced with 1 drop of ferric chloride and a deep blue color is formed with 3 or 4 drops of the reagent.

Attempts to crystallize the glucoside by the method of Willstätter and Zollinger were unsuccessful. At first a few hexagonal-shaped crystals separated from the solution, but for the most part the substance separated, after the solution had stood for several days, in spherical granules. A sample of this precipitate was analyzed after it had been dried at 105° in a vacuum over phosphorus pentoxide. The loss in weight on drying was 5.98 per cent, corresponding nearly to 2 molecules of water of crystallization. Calculated for 2 H_2O: H_2O 6.37 per cent.

Analyses. 0.2039 gm. substance: 0.0890 gm. H_2O and 0.3945 gm. CO_2. 0.2906 and 0.3142 gm. substance: 0.0941 and 0.1032 gm. AgCl.

Calculated for anthocyanin chloride, $C_{23}H_{25}O_{12}Cl$ (528.5). C 52.22, H 4.73, Cl 6.71 per cent.

Found. C 52.75, H 4.89, Cl 8.00, 8.11 per cent.

The percentages of carbon and chlorine were too high and the fact that we found in the quantitative hydrolysis values too high for anthocyanidin chloride and too low for glucose makes it very probable that partial hydrolysis had occurred during the long contact with the acidified alcohol.

Quantitative Hydrolysis of the Glucoside.

The substance used in this determination had been purified as follows: The crude anthocyanin chloride was dissolved in water, acidified with a few drops of dilute hydrochloric acid, and precipitated by adding a warm saturated solution of picric acid. The picrate was filtered and recrystallized from water. The substance was filtered, dried, dissolved in methyl alcohol containing dry hydrochloric acid, and precipitated with ether. The amorphous substance was filtered, washed free from picric acid with ether, and dried in a vacuum desiccator over sulfuric acid. This amorphous anthocyanin chloride, 0.4774 gm., was dissolved in 12 cc. of water, 13 cc. of concentrated hydrochloric acid were added, and the solution was boiled for 4 minutes. The crystals of anthocyanidin chloride were filtered after the solution had cooled, washed with dilute hydrochloric acid, and allowed to dry in the air. The yield was 0.3275 gm. The small amount of pigment that remained dissolved in the filtrate was extracted with 3 portions of amyl alcohol. This extract was diluted to 250 cc. and compared in a colorimeter with a standard solution. It was found to contain 0.0183 gm. of pigment. The total amount of anthocyanidin chloride obtained, accordingly, weighed 0.3458 gm. The theoretical amount of anthocyanidin chloride, calculated for $C_{17}H_{15}O_7Cl + 1.5\ H_2O$, is 0.3554 gm. The amount of pigment recovered is, therefore, 97.3 per cent.

The aqueous solution, mentioned above, that had been extracted with amyl alcohol was used for the determination of glucose after first extracting the amyl alcohol with ether. In all these operations the extracting solvent was washed with water to minimize loss of glucose. The clear solution was neutralized with sodium hydroxide and boiled with 60 cc. of Fehling's solution. The cuprous oxide weighed 0.2859 gm. which is equivalent to 0.1314 gm. of glucose. This is 82 per cent of the theoretical amount, 0.1626 gm.

Identification of the Glucose.

The filtrate obtained after hydrolyzing 6 gm. of anthocyanin chloride was extracted with amyl alcohol until all the color was

removed. The solution was treated with an excess of lead carbonate and the lead salts were filtered off. The filtrate was treated with hydrogen sulfide to remove excess of lead and the lead sulfide was filtered off. The solution was neutralized with sodium hydroxide and concentrated under reduced pressure to about 30 cc. It was of a pale yellow color.

In a 1 dm. tube the angle of rotation was +2.76°. 3 cc. of the solution gave 0.3702 gm. of cuprous oxide on boiling with Fehling's solution which is equivalent to 0.1724 gm. of glucose. Hence, $[\alpha]_D^{20} = +48.0°$. This corresponds quite closely to the specific optical rotation of glucose which is +52.8°.

The balance of the solution, 6.4 cc., was diluted to 30 cc. with water and heated in a beaker of boiling water. To the hot solution were added 3.5 cc. of an aqueous solution containing 0.67 gm. each of phenylhydrazine and glacial acetic acid. Crystals of the osazone began to separate after 11 minutes. These crystals had the characteristic appearance of phenylglucosazone and after the substance had been recrystallized from dilute alcohol it melted with decomposition at 206° (uncorrected). The time of osazone formation and the melting point correspond closely to that of phenylglucosazone.

Preparation and Properties of Anthocyanidin Chloride.

The sugar-free pigment is easily formed when the glucoside is boiled with about 20 per cent hydrochloric acid. The glucoside, 3 gm., was dissolved in 37 cc. of water and the solution was filtered. After adding 38 cc. of concentrated hydrochloric acid the solution was boiled for 4 minutes. The anthocyanidin chloride began to crystallize after the solution had been boiled about 2 minutes. After the solution had cooled the substance was filtered, washed with dilute hydrochloric acid, and dried in the air. The yield was about 1.2 gm.

In crystal form and in color the substance cannot be differentiated from the pigment isolated from Norton and Concord grapes as described in the first paper.[1] The crystals dissolve in water giving a dull brownish red solution which becomes bright red when acidified and blue when made alkaline. An aqueous solution of the pigment is decolorized immediately by ferric chloride, but when

dissolved in ethyl alcohol the addition of a small quantity of ferric chloride produces a momentary purple color which changes quickly to blue, but in the course of a few hours the blue color fades leaving a nearly colorless solution. In this color reaction with ferric chloride the substance differs from the anthocyanidin from Norton and Concord grapes and the reaction is probably due to a difference in position of the methoxyl groups in the gallic acid part of the molecule.

The substance was dried for analysis at 105° in a vacuum over phosphorus pentoxide. The loss in weight corresponds to 1.5 molecules of water of crystallization.

Analyses. 0.1832 and 0.1845 gm. substance lost on drying 0.0131 and 0.0139 gm. 0.1701 and 0.1706 gm. substance: 0.0592 and 0.0603 gm. H_2O and 0.3437 and 0.3463 gm. CO_2. 0.2126 gm. substance: 0.0799 gm. AgCl.

Calculated for $C_{17}H_{15}O_7Cl$ (366.5). C 55.66, H 4.09, Cl 9.68 per cent.

Found. C 55.11, 55.36; H 3.89, 3.95; Cl 9.29 per cent.

Calculated for 1.5 H_2O. H_2O 6.86 per cent.

Found. H_2O 7.15, 7.53 per cent.

These analyses correspond quite closely to the composition of a dimethyl ether of delphinidin, but it was found by the Zeisel method that there was present only slightly more than one methoxyl group.

Analyses. 0.2185, 0.1928, and 0.6140 gm. substance: 0.1591, 0.1455, and 0.4293 gm. AgI.

Calculated for 2 CH_3O. CH_3O 16.91 per cent.

" " 1 " " 8.45 " "

Found. CH_3O 9.62, 9.97, 9.23 per cent.

Delphinidin Iodide and Chloride.

When the contents of the flasks used in the methoxyl determinations were allowed to cool, delphinidin iodide separated as shining prisms or whetstone-shaped crystals. The crystals were collected on a Büchner funnel, washed with ether, and allowed to dry in the air. The substance had a beautiful bronze luster. The iodide was converted to the chloride by shaking with silver chloride and hydrochloric acid in methyl alcohol solution as described by Willstätter and Zollinger.[2] The residues from the three determinations yielded slightly more than 1 gm. of the iodide from which 0.53 gm. of delphinidin chloride was obtained.

The delphinidin chloride dissolved easily in alcohol giving a beautiful red solution. When the alcoholic solution was diluted with water and warmed on the water bath the color gradually faded leaving finally a colorless solution. Evaporation of a small amount of this solution on a watch-glass left fine needles of the colorless base.

The delphinidin chloride was analyzed after it had been dried in a vacuum over phosphorus pentoxide at 105°. The loss in weight corresponded to 1.5 molecules of water of crystallization.

Analyses. 0.1985 and 0.1633 gm. substance lost on drying 0.0150 and 0.0124 gm. 0.1509 and 0.1469 gm. substance: 0.0479 and 0.0445 gm. H_2O and 0.2948 and 0.2897 gm. CO_2. 0.1827 gm. substance: 0.0770 gm. AgCl.

Calculated for $C_{15}H_{11}O_7Cl$ (338.5). C 53.17, H 3.24, Cl 10.48 per cent.

Found. C 53.28, 53.78; H 3.55, 3.39; Cl 10.42 per cent.

Calculated for 1.5 H_2O. H_2O 7.38 per cent.

Found. H_2O 7.55, 7.59 per cent.

Formation of Phloroglucin by Alkali Fusion.

1 gm. of anthocyanidin chloride was decomposed by heating with a solution of 10 gm. of potassium hydroxide in 3.5 cc. of water in a tube through which hydrogen was passed. The tube was heated in an oil bath and the pigment was added when the temperature had reached 160°. The heating was continued for 10 minutes during which time the temperature had risen to 200°. The color of the melt changed from brown to nearly black.

When the mixture had cooled it was dissolved in 100 cc. of 12.5 per cent hydrochloric acid. The solution was made slightly alkaline with sodium bicarbonate and the phenolic constituents were extracted with ether. The ether was evaporated and the residue was dissolved in water, decolorized with norit, filtered, and the solution was concentrated in a vacuum desiccator over sulfuric acid. Nearly colorless crystals of phloroglucin separated which, after being filtered and dried, melted between 210° and 212°. It gave all the reactions noted in a previous paper.[1]

The bicarbonate solution was acidified and extracted with ether. On evaporation of the ether a dark colored residue remained from which no crystalline material could be obtained. It gave a dirty blue-black precipitate with ferric chloride.

Acetylation of Anthocyanidin Chloride and Oxidation.

2 gm. of anthocyanidin chloride were boiled for 4 hours with 25 cc. of acetic anhydride. The color changed to brown and a dark colored amorphous precipitate formed. The acetic anhydride was distilled off under reduced pressure and the residue was dissolved in 100 cc. of alcohol. The acetyl derivative was precipitated as a flocculent amorphous substance by the addition of 350 cc. of water. It could not be obtained in crystalline form from any of the usual solvents. The dried substance melted unsharply between 205° and 210°.

The crude substance was oxidized directly with neutral permanganate. It was suspended in water and to it were added 10 gm. each of potassium permanganate and magnesium sulfate. The mixture was stirred at room temperature for several hours until the color of the permanganate had practically disappeared. The mixture was then acidified, treated with sodium bisulfite until colorless, filtered, and extracted with ether. The ether was evaporated and the residue was dissolved in water and decolorized with norit. The solution was concentrated in a vacuum desiccator over sulfuric acid. The residue consisted of nearly colorless crystals mixed with some amorphous sticky substance. It was possible to separate this material by extracting it with cold benzene which dissolved only the crystals. The benzene was evaporated and the residue was recrystallized from water. The white granular substance weighed 0.2 gm. It melted between 144° to 146° (uncorrected).

The aqueous solution of the substance showed an acid reaction to litmus. This solution gave no coloration with ferric chloride. After the substance had been hydrolyzed by boiling with concentrated hydrochloric acid and the acid had been evaporated the aqueous solution gave with dilute ferric chloride a blue-black coloration similar to that given by gallic acid itself.

For analysis the substance was dried at 105° in a vacuum over phosphorus pentoxide, but it did not lose in weight.

Analysis. 0.1092 gm. substance: 0.0459 gm. H_2O and 0.2191 gm. CO_2. Found. C 54.72, H 4.70 per cent.

It will be noticed that these values are intermediate between the calculated composition of a dimethyl ether of monoacetylgallic acid, $C_{11}H_{12}O_6$ (240), C 55.00, H 5.00 per cent, and a monomethyl ether of diacetylgallic acid, $C_{12}H_{12}O_7$ (268), C 53.73, H 4.47 per cent.

In the Zeisel determination an intermediate value was also obtained as is indicated in the figures given below.

Analysis. 0.0875 gm. substance: 0.1108 gm. AgI.
Found. OCH_3 16.73 per cent.
Calculated for 2 OCH_3. $O.CH_3$ 25.83 per cent.
" " 1 " " 11.56 " "

The analyses indicate that the substance is a mixture consisting approximately of 40 per cent of a dimethyl ether of monoacetylgallic acid and 60 per cent of a monomethyl ether of diacetylgallic acid.

Identification of Gallic Acid.

The residue from the above mentioned Zeisel determination was diluted with water and treated with sodium bisulfite to remove any free iodine and it was then extracted with ether. The ether was evaporated and the residue was dissolved in water, decolorized with norit, and the solution was concentrated. Fine needles of gallic acid separated when the solution had cooled. It was recrystallized from water, filtered, and dried. The substance melted with decomposition at 243°. Some carefully recrystallized gallic acid melted with decomposition at the same temperature and in appearance both substances were identical. With ferric chloride the substance gave a blue coloration and later a blue-black precipitate formed. On adding a solution of potassium cyanide a pink color was obtained that gradually faded to yellow, but the pink color was restored when the solution was shaken. The substance reduced Fehling's solution. The melting point and the above mentioned reactions show that the substance was gallic acid.

Absorption Spectra of Anthocyanin and Anthocyanidin Chloride.

The following absorption bands were observed.

Anthocyanin Chloride. 1 Molecule in 2,000 Liters.

Column.	Methyl alcohol.	Ethyl alcohol.
mm.		
2	575 .. 555 509	583 .. 561 .... —
3	577 .. 559 503	585 .. 566 505
5	578 .. 562 498	587 .. 571 500
7.5	583 .. 570 488	590 .. 575 493
10	587 .. 573 480	595 .. 584 492

Anthocyanidin Chloride. 1 Molecule in 2,000 Liters.

Column.	Methyl alcohol.	Ethyl alcohol.
mm.		
1	575 .. 553 520	581 .. 562 551
2	581 .. 562 514	587 .. 567 527
3	584 .. 567 507	590 .. 571 509
5	590 .. 574 485	594 .. 580 494
10	595 .. 577 .... —	596 .. 589 .... —

SUMMARY.

The pigment occurring in Clinton grapes consists principally of a monoglucoside, anthocyanin. The anthocyanin chloride, $C_{23}H_{25}O_{12}Cl$, did not separate in definitely crystalline form, but the picrate, $C_{23}H_{24}O_{12}\cdot C_6H_2(NO_2)_3OH$, crystallized in bright red prisms or needles. The glucoside is easily hydrolyzed by boiling dilute hydrochloric acid yielding 1 molecule each of glucose and anthocyanidin chloride, $C_{17}H_{15}O_7Cl$, the latter crystallizing from the hot solution in prisms.

The anthocyanidin chloride consists largely of a monomethyl ether of delphinidin, but the values for methoxyl were too high, indicating that it contained some dimethyl ether of delphinidin.

The absorption spectra of anthocyanin chloride and anthocyanidin chloride consist of one broad band extending from the yellow into the blue.

A STUDY OF THE EFFECT OF TEMPERATURE ON PROTEIN INTAKE.

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(Received for publication, April 26, 1924.)

There is a wide-spread popular impression that less food, and particularly less protein, is eaten during warm than during cold weather, and, furthermore, the opinion is frequently expressed that in the tropics a lower food intake is needed than in the countries of the north.

The experimental and statistical work which has been carried out on this phase of the nutrition problem has been admirably discussed in considerable detail by Greenwald (1) who summarizes the situation in the following terms: "It is a generally accepted belief that less food is required in summer than in winter and less in the tropics than in temperate climates. But there are very few accurate observations and such as there are do not support this belief." As an extremely complete bibliography is furnished by Greenwald we will here omit references to the early work on this subject.

Some years ago we were struck by the low figures for urinary nitrogen obtained by medical students at this Institution, who were carrying on metabolism experiments on themselves as a part of the routine laboratory work in the course in biological chemistry. Our experience in this type of experimental work, gained at one of the large New England medical schools, had led us to expect figures for total urinary nitrogen in the 24 hour urines of active young men to be in the neighborhood of 15 to 18 gm., whereas our students, almost without exception reported values of not more than half this amount.

Such results were, of course, viewed with suspicion, but as calculation of the creatinine coefficients (2) gave figures which were

well within the normal limits, and when several 24 hour collections gave similar creatinine figures, we were compelled to conclude that we were not dealing with cases of incomplete urinary collection, but with dietary habits which were not in accord with the accepted normal average.

In order to obtain more comprehensive figures on the subject, we have, during a period of 3 years, collected data on the 24 hour excretion of total urinary nitrogen and creatinine by medical students who were eating their customary diet, at the University dining hall, at boarding houses, fraternity houses, or at their own homes, and who were entirely ignorant of the nature of our inquiries, although they were trained in the technique of accurate urine collections, and were aware of the fact that by means of the creatinine values we were in a position to check the accuracy and faithfulness of their collections. After the elimination of the material supplied by a few subjects who, we had reason to believe, were not trustworthy, it was found that we were in possession of figures obtained on 233 men and 9 women. The number of women is so small that the figures obtained for them have been omitted from the average results recorded in Table I which, therefore, apply only to our male subjects.[1]

Our material was obtained during the month of April in 1922 and 1923 and during July, 1923. In 1924 the experiments were carried on from February 20 to March 6. Our nitrogen determinations were made by the Kjeldahl method and the creatinine by the modified micro technique of Folin (3) using creatinine zinc chloride as a standard.

The figures for temperature were taken from the monthly report of the local office of the United States weather bureau.

The age of our subjects was found to be mainly between 18 and 22 years, together with a few older men, the oldest being not more than 30.

[1] The figures obtained on nine women were as follows:

	Average.	Maximum.	Minimum.
Urinary nitrogen, *gm*	7.72	10.62	4.05
Creatinine coefficient	7.37	8.50	5.97
Body weight, *kg*	55.50	65.91	45.00

Our students come chiefly from the Southern States, so that it would appear that food habits in this group must be of a distinctly localized variety.

An inspection of the data collected in Table I shows that the average 24 hour excretion of urinary nitrogen by our entire group of 233 subjects amounted to 10.63 gm. or, if calculated to the 70 kilo man equivalent, to 11.07 gm.

10.63 gm. of nitrogen are equivalent to 66.43 gm. of protein, and if we make the commonly accepted assumption that approximately 10 per cent of the food protein is lost in the feces this would

TABLE I.

Total Nitrogen Excretion in Urine and Creatinine Coefficients Averaged According to Years in Which the Observations Were Made.

Year.	No. of subjects.	Average weights.	Average urinary nitrogen per 24 hrs.	Average creatinine coefficient.	Temperature.		
					Mean.	Maximum.	Minimum.
		kg.	*gm.*		°C.	°C.	°C.
1922	62	66.80	10.27	9.01	22.94	26.83	19.06
1923	75	67.35	10.64	8.79	21.00	25.33	16.66
1923 (Summer).	18	64.86	9.43	8.79	26.77	30.66	22.94
1924	78	68.00	11.18	9.03	13.05	17.33	8.83
Average.....	233	67.23	10.63	8.93			
Average per 70 kg. man equivalent..			11.07	9.30			

bring the total average protein intake of this group to 73.8 gm., a figure considerably below the average of 121 gm. which Pearl (4) has recently calculated to represent protein intake in the United States and approximately half as large as the 150 gm. "American Standard" protein diet of Atwater.

The effect of temperature on protein intake is well shown in Table I and in Chart 1, for we were fortunate enough to be able to make observations during a period of midsummer weather, during the coldest weather of an abnormally cold winter, and during the intermediate temperatures which prevail in April.

The constant fall in nitrogen excretion with increase in temperature as shown in Chart 1 is in striking contrast to the small deviation from the horizontal given by the curve for the creatinine coefficients which, for comparison, are plotted on the same chart.

In Chart 2 are presented the results of an analysis of the distribution of nitrogen excretion; this chart brings out the rather interesting fact that 39.49 per cent of our subjects excreted between 9.00 and 10.99 gm. of urinary nitrogen in 24 hours,

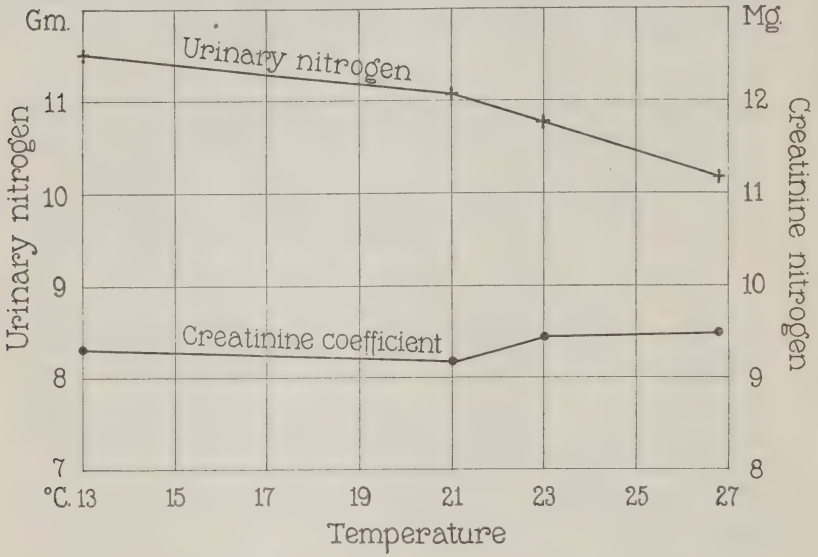
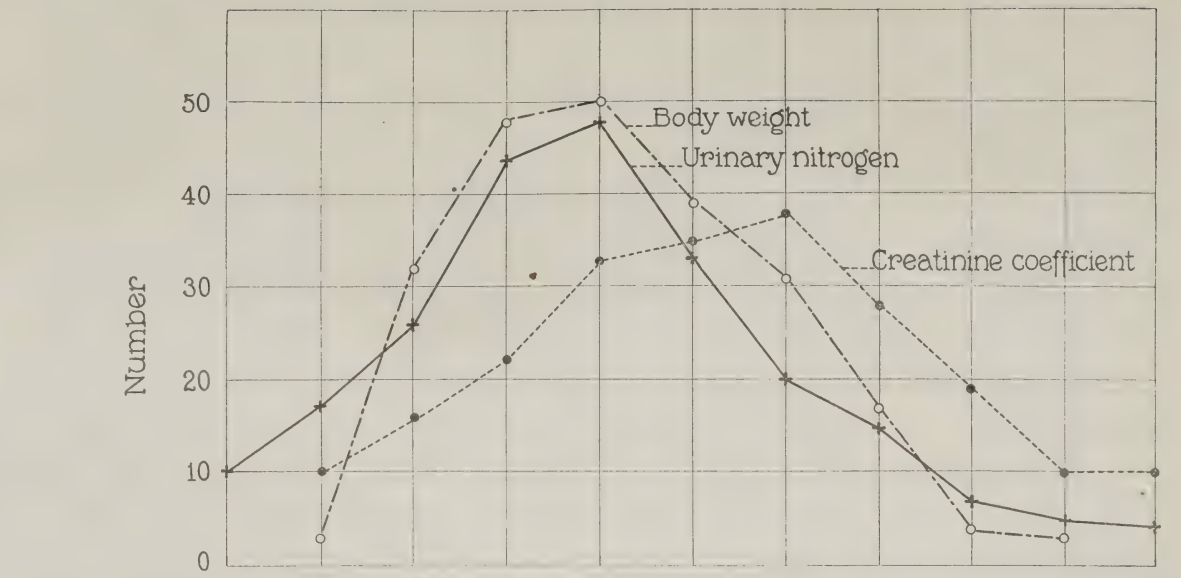


Chart 1. Showing effect of temperature on urinary nitrogen and creatinine coefficient, calculated to 70 kilos of body weight.

indicating (if we add 10 per cent of the urinary loss for fecal nitrogen) an intake of 62.5 to 76.3 gm. of protein, while only 30 per cent (7 men) were in the division which excreted 14 to 14.99 gm., of which 4 subjects were in the group taken during the cold weather of February and March, 1924. For comparison we have also shown in Chart 2 the distribution of weight and creatinine coefficients of our subjects.

As stated above, our subjects were eating either at boarding houses, fraternity houses, in their own homes, or at the University dining hall. On the whole it may be said that the average financial standing of each group was about the same. There was but



Urinary nitrogen	6	7	8	9	10	11	12	13	14	15	16
Creatinine coefficient		6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0
Body weight		110	120	130	140	150	160	170	180	190	

CHART 2. Showing the distribution of urinary nitrogen, creatinine coefficient, and body weight.

little difference in the nitrogen excretion of these four classes, the highest (11.25 gm.) was given by the fraternity house group (44 subjects, average body weight 68.50 kilos, and average creatinine coefficient 8.81 mg. per kilo of body weight), the second (11.01 gm.) by the boarding house group (86 subjects, average body weight 68.21 kilos, and average creatinine coefficient 8.94 mg. per kilo of body weight), the third (10.80 gm.) by the home group (55 subjects, average body weight 63.38 kilos, and average creatinine coefficient 9.00 mg. per kilo of body weight), and the fourth and lowest (9.45 gm.) by the group who ate at the University dining hall (50 subjects, average body weight 66.4 kilos, and average creatinine coefficient of 8.96 mg. per kilo of body weight).

The data have been analyzed by dividing the results into groups arranged according to the place of residence of the subjects, as it appeared possible that persons who had been accustomed to a colder climate might have continued their food habits when transplanted to a new environment. We have therefore classified our results into five groups, a Gulf State group (180 subjects, average body weight 66.76 kilos, average urinary nitrogen 10.80 gm., and average creatinine coefficient 8.91 mg. per kilo of body weight) which included all individuals whose homes were in the states of Florida, Alabama, Mississippi, Louisiana, and Texas; second, a southern group (34 subjects, average body weight 69.83 kilos, average urinary nitrogen 10.33 gm., and average creatinine coefficient 8.81 mg. per kilo of body weight) which contained those from Georgia, North and South Carolina, Tennessee, Kentucky, Missouri, Arkansas, and Oklahoma; third, a group (8 subjects, average body weight 67.80 kilos, average urinary nitrogen 12.51 gm., and average creatinine coefficient 9.90 mg. per kilo of body weight) from states north of the Mason-Dixon line and east of the Rocky Mountains; fourth, a group (7 subjects, average body weight 67.21 kilos, average urinary nitrogen 10.14 gm., and average creatinine coefficient 9.36 mg. per kilo of body weight) from the states west of the Rocky Mountains; and last, a small miscellaneous group (4 subjects, average body weight 63.75 kilos, average urinary nitrogen 11.39 gm., and average creatinine coefficient 10.35 mg. per kilo of body weight) who did not fit into the above classification and who were chiefly residents of Central and South America, Costa Rica, etc.

This classification has brought out the fact that the group of subjects from the Northern States showed an average excretion of about 2 gm. of nitrogen in excess of the average figure for the groups from the Gulf and Southern States. As, however, the number of subjects in the former group amounted to a total of only eight men whereas the latter contained 214, it would appear unwarranted to assign too much importance to this finding.

SUMMARY.

Analyses of 24 hour urines collected over a period of 3 years from 233 male medical students, who were eating their ordinary diet, at their customary eating places, indicated an average excretion of urinary nitrogen of 10.63 gm. This figure plus 10 per cent added to account for the nitrogen lost through the feces indicates an average consumption of 73.8 gm. of protein, an amount which is distinctly below the average protein intake (121 gm.) recorded for inhabitants of the United States. The relation of low protein intake to poverty is well shown in dietary studies made on special groups of people, but the fact should be emphasized that the subjects of this experiment were all at least comfortably supplied with the necessities of life, many were distinctly well to do, and all were living at eating places where board was paid for on a monthly basis and was supplied in liberal amounts, hence no question of possible economy in the purchase of food need be considered. The suggestion is made that this lowered protein intake may be due to the warm climate in which these subjects were living as it was noted that the average nitrogen of the urines collected during the cold weather of February and March was noticeably higher than in those collected during April and July. The results obtained furnish evidence in favor of the view that increase of temperature is accompanied by a decrease in protein intake, and that, apart from the variations due to seasonal changes, the inhabitants of the semitropical portions of this Country probably consume an amount of protein considerably below the quantity reported as the average intake for the nation.

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SOME NITROGENOUS CONSTITUENTS OF THE JUICE OF THE ALFALFA PLANT.

II. THE BASIC NITROGEN.*

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INTRODUCTION.

Our present knowledge of the simpler nitrogenous substances dissolved in plant juices is exceedingly scanty. Although certain individual compounds have long been known to occur in plants, there is, so far as we can find, no plant which has been thoroughly investigated with the object of learning as much as possible regarding the substances dissolved in its juice, and in no case has any large proportion of the nitrogen of a plant juice been accounted for in a way satisfactory to the organic chemist. The series of papers from this laboratory dealing with this subject is an attempt to do this for the alfalfa plant. They already show the inadequacy of present methods and present knowledge and indicate the existence of a field of investigation hitherto largely neglected by biochemists.

The present investigation was undertaken to ascertain the extent to which the basic substances already found in the hydrolyzed juice of the alfalfa plant occur in the free form rather than as constituents of peptides or other complexes.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C. The writer wishes to express his thanks to Dr. Thomas B. Osborne for his interest in the work and also for much helpful advice and criticism.

Leavenworth, Wakeman, and Osborne (1) recently employed Kossel's method to examine the filtrate[1] obtained after proteins had been precipitated from the juice of the alfalfa plant by means of alcohol. They subjected this filtrate to a severe acid hydrolysis and established the presence in it of notable amounts of arginine, lysine, stachydrine, and a base which yielded a picrate melting at 298°C.

TABLE I.

		Total nitrogen.
	gm.	*per cent*
Total nitrogen	10.06	
Ammonia "	0.4066	4.04
Amide "	0.8344	8.29
Amino "	3.67	36.5
Nitrate* "	0.328	3.25
Other "	4.82	47.91
Solids	175.0	
Ash	28.2	

* Nitrate nitrogen was determined by reduction in alkaline solution with Devarda's alloy and aeration of the ammonia into standard acid. A blank was run at the same time and the ammonia set free by the alkali alone subtracted.

GENERAL DISCUSSION OF THE YIELDS.

1 liter of the "alfalfa filtrate" used gave, on analysis, the results in Table I. This quantity of filtrate is equivalent to approximately 6,460 gm. of fresh or 1,550 gm. of the dried plant.

Table I shows that of the 10.06 gm. of nitrogen, 4.82 gm. belongs to other than the ammonia, nitrate, amide, and amino groups. Less than 0.8 gm. of this nitrogen was accounted for as choline and stachydrine, the purines, or by the non-amino nitrogen of arginine and lysine. After severe acid hydrolysis of a portion of the filtrate, amino nitrogen was found equivalent to 4.91 gm. per liter. Deducting the 3.67 gm. of free amino

[1] In the following pages the term "alfalfa filtrate" designates the fluid prepared from the juice of the alfalfa plant by adding alcohol to a concentration of 53 per cent by weight, concentrating the filtrate from the copious precipitate *in vacuo*, and adding approximately 30 per cent of alcohol. Thus prepared the "alfalfa filtrate" represents about 4 volumes of the original juice of the alfalfa plant and is free from protein.

nitrogen leaves 1.24 gm. set free by the hydrolysis. Assuming that this is a measure of the peptide nitrogen of the alfalfa filtrate there is a residue of about 2.7 gm., or over one-quarter of the nitrogen of the alfalfa filtrate belonging to groups of a wholly unknown nature.

The amounts of the substances listed in Table II were isolated in a state of purity from 1 liter of this filtrate without subjecting

TABLE II.

	Substance.	Nitrogen.
	gm.	*gm.*
Arginine	0.522	0.172
Lysine	0.073	0.014
Stachydrine	3.768	0.371
Choline	0.249	0.036
Free base yielding picrate of melting point 298°	0.290	0.146
Chloride of a purine	0.048	0.014 (estimated).
Previously reported (5):		
Asparagine	3.193	0.596
Tyrosine	0.224	0.0173
Total	8.367	1.3663

it to hydrolysis. These substances therefore occur free, or as salts, in the alfalfa filtrate to at least the extent given.

They account for only 4.8 per cent of the organic solids and 13.6 per cent of the nitrogen of the filtrate.[2]

The picrate melting at 298°, of which 0.88 gm. was obtained, contains approximately 67 per cent of picric acid, and the free base is approximately 50 per cent nitrogen. These figures have been used in calculating the yields in Table II. More accurate results will be given when sufficient material has been obtained for further study.

More stachydrine was found in alfalfa filtrate than any other one constituent. Steenbock (2) obtained a yield of 0.22 per cent of the free base from dry alfalfa, assuming that the hay he

[2] The yields are expressed in terms of the nitrogen and solids of the alfalfa filtrate as a matter of convenience, inasmuch as we are chiefly concerned at this stage with the chemical composition of this material.

analyzed contained 8.6 per cent of moisture (3). The above yield is 0.24 per cent of the dry alfalfa used in this work. Our result is therefore in substantial agreement with Steenbock's.

Leavenworth, Wakeman, and Osborne obtained 4.34 gm. of stachydrine from a quantity of *hydrolyzed* alfalfa filtrate which contained 8.26 gm. of nitrogen and was derived from approximately 1,270 gm. of the dry plant. This is a yield of 0.34 per cent. The higher result is perhaps due to more favorable conditions, since much of the troublesome coloring material was destroyed and removed in the humin resulting from the hydrolysis.

Table III gives the results of Leavenworth, Wakeman, and Osborne on the *hydrolyzed* alfalfa filtrate, together with those obtained in the present work. The quantities are grams of substance per gram of nitrogen in the alfalfa filtrate used.

TABLE III.

	Unhydrolyzed.	Hydrolyzed.
	gm.	*gm.*
Base yielding picrate of melting point 298°.....	0.029	0.009
Arginine.....	0.052	0.099
Lysine.....	0.007	0.060
Stachydrine.....	0.375	0.525

The smaller amount of the base which yields a picrate of melting point 298° obtained after hydrolysis, is probably due to its partial destruction by the boiling acid. It is evident that this base occurs free, or as a salt, in the juice of the alfalfa plant.

At least half the arginine of the alfalfa filtrate occurs uncombined. Unfortunately the mother liquor from which the arginine had been isolated was discarded without submitting it to hydrolysis; consequently it has not been ascertained whether arginine occurs also in peptides.

The small amount of lysine which was isolated before hydrolysis, indicates that very little of this diamino acid occurs free. The fraction which might contain lysine was precipitated repeatedly with mercuric chloride and baryta, following Winterstein's procedure (4), but even so, more lysine could not be isolated. Finally a portion of this fraction was subjected to severe acid hydrolysis whereby approximately one-quarter of its nitrogen was found to have been in the peptide form. After hydrolysis

lysine picrate equivalent to 0.03 gm. of lysine per gram of nitrogen of the filtrate was readily obtained. The greater part of the lysine of alfalfa filtrate doubtless occurs in combination, probably in peptides.

A small amount of choline was found in the mother liquors of the stachydrine hydrochloride. Leavenworth, Wakeman, and Osborne did not examine their solutions for this base.

With regard to the nature of the trace of purine found there is as yet little definite information. It gives the murexide and xanthine tests but does not appear to be identical with xanthine itself or with the commoner purines of this class.

Method of Fractionation.

The basic substances in the alfalfa filtrate are obtained by the methods herein employed in two separate fractions. The diamino acids and purines are precipitated by Neuberg's reagent (mercuric acetate, sodium carbonate, and alcohol), and were subsequently separated with phosphotungstic acid.

The betaines, on the other hand, are not precipitated by Neuberg's reagent, but were recovered from the filtrate from this precipitation either with phosphotungstic acid or acid mercuric chloride. The successive steps of the scheme of fractionation have been given in detail in the previous paper (5). The accompanying outline may assist in following the operations. The figures refer to the steps of the scheme previously given, but all details are omitted.

Outline of the Method of Fractionation.

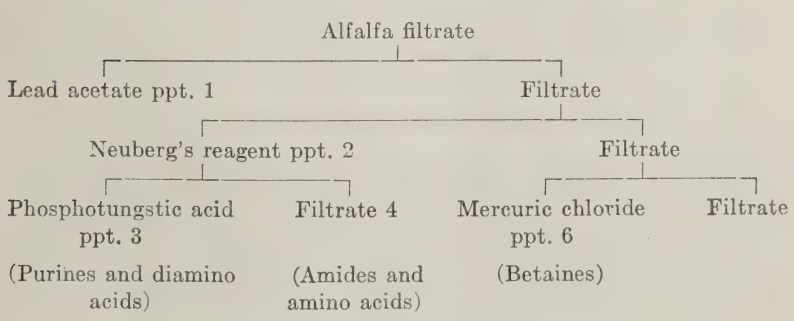

Table IV gives the analysis of the filtrate from the lead acetate precipitate.

Table V gives the analysis of the precipitate obtained from this solution with Neuberg's reagent.

Table VI gives the analysis of the filtrate from the Neuberg reagent precipitate.

TABLE IV.

	From 1,000 cc. alfalfa filtrate.	Of each form of nitrogen in 1,000 cc. of alfalfa filtrate.
	gm.	*per cent*
Total nitrogen	8.52	84.7
Ammonia "	0.125	30.7
Amide "	0.827	99.1
Amino "	2.90	79.0
Other "	4.67	96.9
Solids by difference	147	84

TABLE V.

	From 1,000 cc. alfalfa filtrate.	Of each form of nitrogen in 1,000 cc. of alfalfa filtrate.
	gm.	*per cent*
Total nitrogen	5.81	57.8
Ammonia "	0.054	13.3
Amide "	0.731	87.6
Amino "	2.58	70.0
Other "	2.445	50.7
Solids, ash-free	44.45	25.4

The analysis in Table VI of the filtrate from the precipitate obtained from Neuberg's reagent shows that amides and amino acids had been very completely removed by the reagents. The small amount of amino nitrogen present may be attributed to the solubility of the precipitate since the separation was made in a volume of 8 liters. Approximately one-quarter of the "other nitrogen" was accounted for as nitrogen of stachydrine and choline. Indirect evidence was obtained that a small part of the "other nitrogen" was in the peptide form and the small amount of nitrate nitrogen would doubtless go through to this solution.

There is as yet no evidence whatever regarding the nature of the remaining and greater part of the nitrogen of this fraction.

The large proportion of the solids of the alfalfa filtrate which escape precipitation by the different reagents is worthy of notice. The final solution contains large amounts of alcohol-soluble substances which, although not highly colored, yield intensely colored products on boiling with acid. It may, therefore, prove to be a useful starting point for the investigation of these interesting compounds.

The solution obtained by decomposing the Neuberg reagent precipitate (Table V) was treated with phosphotungstic acid. The analytical data on the precipitate and filtrate thus obtained

TABLE VI.

	From 1,000 cc. alfalfa filtrate.	Of each form of nitrogen in 1,000 cc. of alfalfa filtrate.
	gm.	*per cent*
Total nitrogen	2.377	23.6
Ammonia "	0.009	
Amide "	0.006	
Amino "	0.313	8.56
Other "	2.062	42.8
Solids by difference	92	58

were given in the previous paper (5) and need not be repeated here. The solution of the bases contained 1.95 gm. of nitrogen. Of this only 0.345 gm. was accounted for as nitrogen of arginine, lysine, and the two unknown bases. Apart from a little lysine which could be liberated by hydrolysis, the nature of the remaining nitrogen in this fraction is as yet unknown although a portion doubtless occurs as imino nitrogen of peptides.

EXPERIMENTAL PART.

A. The Filtrate from the Neuberg Reagent Precipitate.—The filtrate from the Neuberg reagent precipitate was acidified with acetic acid, mercury removed with hydrogen sulfide, and the solution concentrated, acidified with hydrochloric acid until acid to Congo paper, and then distilled, under diminished pressure, at as low a temperature as possible, with repeated additions of absolute alcohol, to remove sodium chloride.

Alcohol was then removed and the aqueous solution concentrated to about 300 cc. and excess of hot saturated alcoholic mercuric chloride added. The filtrate from the copious precipitate was concentrated to small volume and again treated with mercuric chloride. The combined precipitates were decomposed with hydrogen sulfide and the solution was concentrated to a sirup under diminished pressure. This was dissolved in a little water. When concentrated over sulfuric acid the sirup solidified to a mass of white crystals which were removed and washed with alcohol. Two more crops were obtained from the mother liquor. The three crops weighed 2.714 gm. and melted with decomposition at 231–232°. Allowing for aliquots previously removed this is equivalent to 3.768 gm. of free stachydrine from 1 liter of the alfalfa filtrate. Once recrystallized from absolute alcohol the substance melted at 235–236° with decomposition, and contained 7.63 per cent of nitrogen. Theory for $C_7H_{13}NO_2HCl$ 7.8 per cent.

The mother liquor of the stachydrine hydrochloride was diluted, and chloride ion removed with silver sulfate. After exact removal of the reagent this yielded an alkaline solution. It was concentrated to small volume, 5 per cent of sodium carbonate added, and treated with Stanĕk's reagent (6). The voluminous precipitate, which rapidly crystallized, was decomposed with finely divided copper and cupric chloride. The solution yielded a precipitate in 50 per cent alcohol with chloroplatinic acid which was recrystallized from dilute alcohol and orange colored octahedra of choline chloroplatinate obtained weighing 0.445 gm., equivalent to 0.249 gm. of choline from 1 liter of the alfalfa filtrate. It melted with decomposition at 237–238°, and contained 30.9 per cent of platinum. Theory for $(C_5H_{14}ONCl)_2PtCl_4$ 31.6 per cent.

The solution from which the betaines had been removed by means of mercuric chloride was freed from reagents, concentrated, and treated with phosphotungstic acid in the usual way. Very little precipitate was obtained and no further stachydrine could be recovered from it. Of the nitrogen in this final solution 36.8 per cent was amino nitrogen. After acid hydrolysis the ratio of amino to total nitrogen was increased to 65.2 per cent, which indicates that nearly 30 per cent of its nitrogen was in the peptide form.

B. The Phosphotungstic Acid Precipitate 3, p. 121.—This precipitate was decomposed in the usual way and hot saturated silver sulfate solution added at an acid reaction. A small flocculent precipitate of a silver salt was centrifuged off, washed with water, decomposed with hydrogen sulfide, and the solution concentrated to dryness under diminished pressure. The crystalline residue was then redissolved in water, and saturated aqueous picric acid solution was added until precipitation was complete. On recrystallizing from hot water a voluminous mass of fine yellow needles separated. The yield was 0.643 gm., equivalent to 0.880 gm. from 1 liter of alfalfa filtrate. Once recrystallized it was obtained as fine sulfur-yellow needles with a silky luster melting sharply with decomposition at 298°. This substance is apparently identical with that reported by Leavenworth, Wakeman, and Osborne (1) in the histidine fraction obtained from hydrolyzed

alfalfa filtrate. The general properties of this base are those of the purines, but the murexide and xanthine tests were negative as was Kossel's test for adenine. The work with this substance has not yet progressed to a point at which a definite statement as to its nature can be made.

The mother liquor from the above picrate on standing for some weeks yielded a few poorly developed dark orange crystals. These were dissolved in water and boiled with norit. The substance could then be obtained, by careful recrystallization, in tiny plates and often in prisms with marked spiral striations entirely different in aspect from the other picrate. The picrate was converted to the chloride and 0.035 gm. of a substance was obtained crystallizing in fine white needles, equivalent to 0.049 gm. from 1 liter of alfalfa filtrate. This chloride was decomposed by water and could only be recrystallized from dilute hydrochloric acid. It gave positive murexide and xanthine tests, but could not be identified with xanthine or any of the commoner purines of this class. This substance is, in all probability, a purine; its identification, however, must await the preparation of quantities sufficient for analysis.

The solution from which the silver sulfate precipitate had been removed was treated in the usual way for the separation of arginine. Analysis of a small aliquot of the solution of the arginine fraction showed the presence of 0.248 gm. of nitrogen. If all this nitrogen belonged to arginine there should have been 0.77 gm. of this diamino acid present. From this solution 1.0273 gm. of arginine picrolonate, equivalent to 0.126 gm. of arginine nitrogen, were obtained. Thus only slightly over one-half of the nitrogen of this fraction was accounted for as arginine. A sample of arginine picrolonate derived from protein, and melting at 241°, was mixed with a recrystallized sample of the picrolonate from alfalfa. The mixture melted at 243°, thus establishing the identity of this picrolonate.

The filtrate from the silver baryta precipitate, when freed from reagents, contained 0.532 gm. of nitrogen. Lysine was separated by Winterstein's method (4) as follows: An excess of mercuric chloride was added and the solution made strongly alkaline with baryta. The precipitate was decomposed with hydrogen sulfide, barium removed, and the solution found to contain 0.462 gm. of nitrogen. Thus 87.7 per cent of the nitrogen in the main lysine fraction has the solubility relations of lysine and would be determined as such by a Kossel analysis of the unhydrolyzed juice in which the operator depended on nitrogen determination rather than isolation for the estimation of the bases. The nitrogen in the mercuric chloride precipitate was equivalent to 0.689 gm. or 6.85 per cent of the nitrogen of the alfalfa filtrate. Consequently the substances in this group are important constituents of the alfalfa juice.

The solution containing the lysine yielded only 0.136 gm. of lysine picrate which decomposed at 256° in the characteristic manner. This yield is equivalent to 0.053 gm. of lysine or to 0.073 gm. based on the alfalfa filtrate. It represents only 3 per cent of the lysine as determined from the nitrogen content of this fraction. Further efforts to obtain more lysine picrate were unsuccessful.

By boiling this solution with 20 per cent hydrochloric acid for 21 hours the ratio of amino to total nitrogen was increased from 50 to 76 per cent. This is indirect evidence of the presence of peptides. In order to determine whether lysine formed a part of such peptides the remaining portion of the hydrolyzed solution was treated with mercuric chloride and baryta as before. From the precipitate so formed 0.205 gm. of very pure lysine picrate, decomposing at 259°, was readily obtained. When allowance is made for aliquots this represents 0.295 gm. of lysine from the alfalfa filtrate, in addition to the amount previously isolated. It should be pointed out, however, that this lysine was very probably in combination whereas that referred to before was found in the free state in the alfalfa filtrate.

Obviously substances other than lysine, but having essentially the same solubility relations, occur in considerable quantities in alfalfa juice. Only about one-fifth of the nitrogen in this final fraction was accounted for as lysine, free and combined. We hope to obtain some chemical evidence of the nature of these other substances in the near future.

SUMMARY.

The proteins and much inorganic matter dissolved in the juice expressed from the fresh ground alfalfa plant were precipitated by the addition of alcohol to a concentration of 53 per cent by weight. The filtrate was concentrated and preserved by the addition of alcohol. The present paper deals with the isolation of certain basic substances from this filtrate.

The method of fractionation employed was reported in a previous paper (5).

The quantities of the substances in the following table were isolated from 1 liter of concentrated alfalfa filtrate which contained 175 gm. of organic solids, and 10.06 gm. of nitrogen, and represented the juice of approximately 6,460 gm. of the fresh plant.

	gm.
Arginine	0.522
Lysine	0.073
Stachydrine	3.768
Choline	0.249
Chloride of a purine	0.048
A base yielding a picrate of melting point 298°	0.290

The isolation of 3.193 gm. of asparagine and 0.224 gm. of tyrosine from this material has been previously reported (5). Together with the above substances these account for only 4.8 per cent of the organic solids and 13.6 per cent of the nitrogen of the alfalfa filtrate.

The above substances have been isolated from the unhydrolyzed filtrate. They, therefore, occur free or as salts, to at least the extent recorded, in the juice of the alfalfa plant.

To the best of our knowledge at the present time all the stachydrine, choline, and the base which yields a picrate melting at 298°, occur free or as salts in the alfalfa juice. Comparison of the present results with those of Leavenworth, Wakeman, and Osborne (1) indicates that arginine is partly in combination and evidence has been obtained that most of the lysine of the alfalfa filtrate is in combination.

Attention has been drawn to the inadequate knowledge of the simpler nitrogenous constituents of the juice of plants and to the desirability of further research in this field.

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THE EFFECT OF COD LIVER OIL IN VARIOUS AMOUNTS AND FORMS ON THE GROWTH OF YOUNG CHICKENS.

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(From Storrs Agricultural Experiment Station, Storrs, Conn.)

(Received for publication, May 29, 1924.)

A number of experiments (1, 2, 3, 4) have shown that cod liver oil is an effective therapeutic agent for the prevention and cure of rickets (leg weakness) in young chickens. We have recently attempted to determine: (1) the minimal amount of cod liver oil necessary for the prevention of rickets in chickens, (2) the effect of one method of storage on the antirachitic potency of cod liver oil, and (3) whether there is any relation between the rate of growth of young chickens and the amount of cod liver oil fed.

EXPERIMENTAL.

Material and Apparatus.—Sixty single combed White Leghorn chickens from our inbred family No. 8[1] were hatched on January 8, 1924. When 24 hours old these chickens were weighed and divided into four equivalent lots. Each lot was placed in one compartment of a laboratory brooder table similar to the one illustrated in Bulletin 116 of this station.[2] This table was rotated a quarter turn each day in order to equalize the amount of light received by the several lots. Heat was supplied by a 30 inch "Electro hatch" hover and the room was kept at about

[1] See *Storrs Agric. Exp. Station, Bull. 111*, for the history for this stock and plan of the inbreeding experiment.

[2] For the first 3 weeks of the experiment it was necessary to use an improvised platform of boards in place of the rotating table illustrated. This platform was rotated one-quarter turn every 2nd day in order to equalize the amount of light received by the several lots. Windows were kept closed throughout the experiment.

60°F. The table was covered with sheet zinc, on which was spread a thin layer of coarse washed sand. No other litter was provided.

Feeding.—When the chickens were 48 hours old they were given pasteurized skim milk (sweet) as drink and a light feeding of fine cracked white corn. From the 2nd until the 7th day they were fed sparingly with white cracked corn, and had skim milk before them at all times. When 7 days old they were again weighed after which the differential feeding of the four lots was begun.

The basic ration adopted for all lots was the formula devised by Hart, Halpin, and Steenbock (1) and found by them to be adequate for growth when supplemented with cod liver oil. It consisted of: white corn meal, 97 parts; calcium carbonate, 2 parts; and sodium chloride, 1 part. This ration may be regarded as practically free from the fat-soluble and antirachitic vitamins. To this we added, as roughage, 10 per cent of sifted pine sawdust, in place of the shavings used as litter by Hart and Steenbock. Supplements were added to this ration for the several lots as follows:

Lot 1.....0.5 per cent cod liver oil (Harris)[3]
" 2.....1 " " " " " (")[3]
" 3.....2 " " " " " (")[3]
" 4.....1 " " " " " (") which had been absorbed in starch, granulated, and held in corked bottles in the dark at about 50°F. for 6 months.

The proportions of supplements were calculated on the basis of the corn meal-salt mixture exclusive of the roughage. In mixing, the oil was poured thinly over the meal and rubbed in thoroughly by hand. The mixture of cod liver oil and starch was pulverized and thoroughly incorporated with the meal. These rations were mixed fresh every 3 days, and fed in small hoppers *ad libitum*. Skim milk was kept before all the lots throughout the experiment.

Growth.—The weekly average weights of these four lots from 0 to 7 weeks are given in Table I and shown graphically in Fig. 1.

[3] The oil used had been stored in unopened corked bottles in the dark at about 50°F. for 6 months.

It will be noted that growth in all the lots was equal through the 3rd week, at which time the rate of growth of Lot 4, receiving dry stored cod liver oil, began to fall behind that of the three other lots.

Rickets.—This decrease in the rate of growth in Lot 4 coincided with and was probably the result of the onset of rickets in this lot. The first symptoms of rickets in Lot 4 were noted when

TABLE I.

Average weights of chickens reared in the laboratory on a basic ration of skim milk, white corn meal, and sawdust, supplemented by various amounts of cod liver oil.

Age.	Lot 1. 0.5 per cent cod liver oil.		Lot 2. 1 per cent cod liver oil.		Lot 3. 2 per cent cod liver oil.		Lot 4. 1 per cent cod liver oil stored in starch 6 mos.	
	Weight.	No. surviving.	Weight.	No. surviving.	Weight.	No. surviving.	Weight.	No. surviving.
wks.	*gm.*		*gm.*		*gm.*		*gm.*	
0	33.0	13	32.9	13	33.3	13	33.2	13
1	37.8	13	37.6	13	39.2	12	38.8	12
2	58.2	13	59.3	13	58.0	12	60.0	12
3	82.3	13	82.8	13	82.5	12	78.7	12
4	110.6	13	109.8	13	113.1	12	94.2	11
5	138.9	13	136.8	13	144.9	11	115.0	11
6	176.7	13	168.4	12	172.9	11	137.9	11
7	210.2	13	207.6	12	204.4	11	171.8	11
7*	215.9	13	214.5	12	219.2	11	173.2	11

* Weights at 7 weeks reduced to male basis, according to the numbers of males and females in each lot.

the chickens were 16 days old. The symptoms of unsteady gait, lameness, apparent stiffness, and swelling of the leg joints appeared then in one chicken, but by the night of the 19th day had become so general and marked that several chickens could not walk to the feed hopper. These chickens (seven in number) were then (20th day) given cod liver oil by pipette at the rate of about 2 drops a day and improvement was noted in each one. Less severe cases were not given cod liver oil, and on the 23rd day four of the five remaining were prostrated.[4] The four pros-

[4] One untreated chicken showed no symptoms of rickets through the 25th day.

trated chicks were then given 2 drops each of cod liver oil, and three of them recovered the use of their legs and were eating normally on the 25th day. One completely prostrated chicken died soon after treatment, possibly from oil which had entered the trachea. All other chickens recovered. On the 26th day administration of oil by pipette was discontinued, and cod liver oil was added to the ration as 2 per cent of the meal-salt mixture. After the 28th day, no further symptoms of rickets were noted in this lot and they continued to grow at rates approxi-

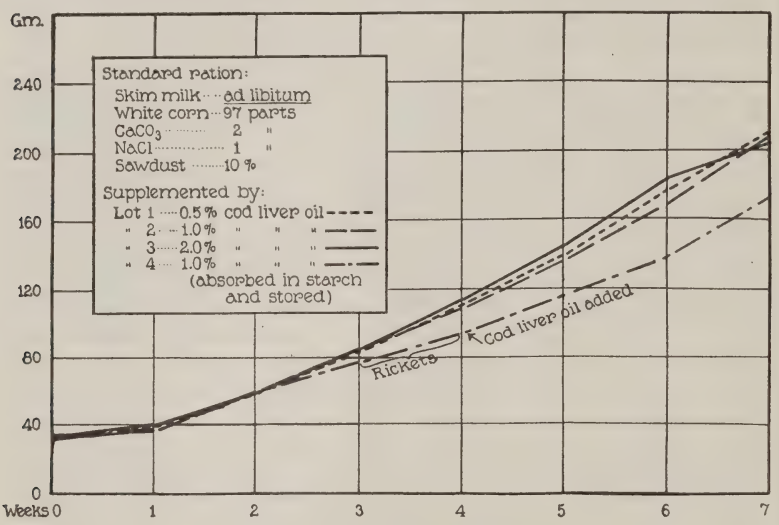

Fig. 1. Comparison of growth of chickens on a standard ration free from the fat-soluble and antirachitic accessory factors supplemented by various amounts of cod liver oil.

mating those of the other lots, although they did not recover the weight lost during the attack of leg weakness. No symptoms of rickets were noted in any of the other lots. It is apparent then that cod liver oil absorbed in starch and stored as described had lost its antirachitic potency; while unmixed oil held for the same time and under the same conditions was both a preventive and a cure for rickets.

Mortality.—In addition to the one death noted in Lot 4, four other chickens died during the course of the experiment. One

chicken in each of Lots 3 (2 per cent cod liver oil) and 4 (1 per cent cod liver oil in dry mixture) died when 1 week old. This was before differential feeding of the lots began and death was probably due in each case to crowding or chilling. One chicken in Lot 2 (1 per cent cod liver oil) died in the 6th week apparently from deposition of fat in the pericardium. It showed no antemortem or postmortem symptoms of rickets. One chicken in Lot 3 (2 per cent cod liver oil) died in the 5th week. Cause of death could not be ascertained. All other chickens grew well and, with the exception of one death from impaction of the crop, are now alive (14 weeks old). The males have been successfully maintained in the laboratory on the ration described above, supplemented by cod liver oil in amounts of from 0.125 to 1 per cent of the ration.

Comparison of Growth on Various Amounts of Cod Liver Oil.— The rations described above were continued until the chickens were 7 weeks old. The graphs in Fig. 1 show that the growth of the chickens receiving 0.5, 1, and 2 per cent of cod liver oil was approximately equal through the 7th week. The graphs illustrating the growth of the different lots are not directly comparable one with another, because of the different proportions of male and female chickens in the several groups. It is known that male chickens gain weight at a somewhat greater rate than females.[5] From other data it has been estimated that the weights of male and female White Leghorn chickens of our stock are about equal up to 3 weeks of age. Thereafter, the females gain less rapidly than the males, until at 7 weeks of age the female weight is about 90 per cent of the male weight. In order to correct for this difference all the 7th week weights have been reduced to the male basis according to the number of males and females in each group. The comparison of the weights so corrected is shown in the last line of Table I. It is evident that there are no significant differences in weight between the lots which received 0.5, 1, and 2 per cent of cod liver oil.

The corrected weight of Lot 4 which had received the starch-stored cod liver oil was significantly less than that of the other groups. Later weighings for 6 weeks showed that this group

[5] Jull (5) and unpublished data at this station.

failed to make up the weight lost during the attack of leg weakness, even when transferred to a normal ration supplemented by cod liver oil.

The Minimal amount of cod liver oil which is necessary to prevent rickets during the first 7 weeks is shown by the above results to lie below 0.5 per cent of the food intake and additional amounts in excess of 0.5 per cent appear to yield no further antirachitic or growth-promoting effects. The actual amounts of cod liver oil consumed by the chickens which received cod liver oil as 0.5 per cent of their solid food intake are as follows:

Age.	Cod liver oil per chicken per day.
wks.	*mg.*
1	0
2	22
3	43
4	56
5	57
6	81
7	89

The minimal amounts necessary apparently lie below the amounts as given.

We have obtained other evidence which indicates that the minimal amount lies between 0.5 and 0.25 per cent of the solid ration for chickens less than 6 weeks old. A group of fourteen chickens from the same family as those in the previous experiment was hatched on January 31, 1924, and was treated and fed like those above except that they received cod liver oil as 0.25 per cent of their meal-salt ration. Symptoms of rickets appeared in this lot on the 26th day, but were confined to half of the chickens in the group. Cod liver oil by pipette effected a cure of this condition and no mortality was experienced until the 6th week, when several chickens died although apparently not from rickets. Because of the individual variation in the occurrence of rickets and the mortality which later occurred we prefer to limit our conclusions from this group to the inference that cod liver oil as 0.25 per cent of the ration is near the minimal amount. It will probably be impossible to measure accurately

the minimal preventive dose of cod liver oil with the methods of group feeding which we have employed because of the individual variation in food consumption and consequently in the amount of oil ingested. For practical purposes we may say that the minimal amount lies between 0.5 and 0.25 per cent of the dry ration. This means that the actual amounts of cod liver oil required by the average chicken is between 25 and 50 mg. per day at 4 weeks of age, the age of maximum incidence of rickets under the conditions outlined above. Other evidence shows that this requirement is subject to variation among individuals, and varies also with age and several factors of the environment.

Experiments designed to measure cod liver oil requirements of older chickens, and their ability to store the antirachitic substance, are now in progress.

CONCLUSIONS.

We interpret the results set forth above as showing:

1. That cod liver oil fed as 0.5 per cent of a ration which is free from the fat-soluble and antirachitic substances, but which contains proper nutrients, salts, water-soluble vitamin, and about 10 per cent of fiber, is sufficient to prevent rickets in chickens reared in strict confinement indoors. The minimal average requirement of the chickens used appears to be between 0.25 and 0.5 per cent of the ration for the first 6 weeks.

2. Addition of increments of cod liver oil above 0.5 per cent of the ration does not increase either the growth rate or protection against rickets.

3. The antirachitic potency of cod liver oil deteriorates or disappears after storage in a granulated starch mixture.[6]

The successful maintenance of a number of chickens in strict confinement in the laboratory, with growth approximating that attained by the same stock out of doors, and a mortality which is much lower than that usually encountered under range conditions, is a further demonstration of the possibility and economy of rearing chickens in confinement, both for scientific and for practical purposes.

[6] A test of this preparation by Dr. T. B. Osborne in feeding experiments with rats showed that the antixerophthalmic potency of the oil had also disappeared under this method of storage (personal communication).

We wish to acknowledge our indebtedness to Dr. T. B. Osborne for his kindly cooperation and advice throughout these experiments, and to Dr. I. F. Harris for his advice and kindness in the preparation of some of the materials used.

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A NOTE ON THE PRESENCE OF VALINE IN ZEIN.

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(Received for publication, June 6, 1924.)

In a recent paper (1) the writer recorded some results of the analysis of the amino acids derived from zein. While in the main close agreement was observed between the new figures and those previously recorded by Osborne and his coworkers (2, 3) certain differences were noted among which was the writer's failure to detect valine. While the writer expressly disavowed the view that his results controverted those of Osborne as the following quotation shows: "Die positiven Funde Osbornes in betriff der Anwesenheit von Valine and Serin sollten in anbetracht der grossen Erfahrung Osbornes auf diesem Arbeitsgebeit nicht in Frage gestellt werden," it appeared desirable to both Osborne and the writer to endeavor to settle the point, especially as zein is frequently used in nutrition experiments in which the presence or absence of any particular amino acid is of importance. Accordingly the following joint experiment was made.

Dr. Osborne undertook the hydrolysis of 250 gm. of thoroughly purified zein, with sulfuric acid (300 gm.) and water (650 cc.). The mixture was boiled for 18 hours and no subsequent heating under pressure was employed. After removal of the sulfuric acid the amino acids were worked up by the writer in precisely the same fashion as that described in the paper already quoted, the details of which need not be repeated. The "leucine valine" fraction was submitted to careful crystallization and was resolved into four fractions of which the first three were almost optically pure *l*-leucine ($[\alpha]_D = 16.4 - 16.9°$ in 20 per cent HCl). The fourth fraction had a rotation of $-18.2°$ and on recrystallization from weak alcohol it rose to $-22.7°$, indicating a mixture of leucine and valine in which the latter was slightly in excess. After taking out a fraction as lead salt according to the method of

Levene and Van Slyke (4), fairly pure valine was easily obtained from the filtrate. The amount of valine actually isolated was 2.1 gm., equivalent to about 1 per cent of the zein, but undoubtedly some was lost. The valine melted at 310–313°, had a specific rotation of +26.9° in 3 per cent solution in hydrochloric acid, and gave satisfactory results on combustion.

Found.	C 51.4, H 9.32, N 11.9.
Calculated.	" 51.2, " 9.40, " 12.0.

There can be no doubt, therefore, that Osborne's original statement as to the presence of small amounts of valine in zein is correct. The writer's failure to isolate it in the first instance may not improbably be ascribed to racemization during the heating in autoclave for 8 hours at 135°, which was employed to insure complete hydrolysis. Even a moderate degree of racemization would introduce serious difficulty in the use of optical methods for the detection of small amounts of valine.

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THE FORMATION OF *l*–MALIC ACID AS A PRODUCT OF ALCOHOLIC FERMENTATION BY YEAST.

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The occurrence of malic acid in fermented products derived from various fruit juices is common knowledge and it has been generally supposed that this substance was exclusively derived from malic acid preformed in the fruit. The object of the following communication is to establish the fact that *l*-malic acid is a true product of fermentation by yeast and that significant amounts of the acid are formed when pure yeast cultures ferment pure sugar solutions. Experiments are also recorded in which an endeavor is made to ascertain the origin of the malic acid and also to determine some of the conditions affecting its production.

The experiments were originally undertaken with the object of obtaining additional evidence as to the structure and stereochemical configuration of the amino acid isolated from various proteins and regarded as β-hydroxyglutamic acid (1). It was thought that in view of the well established formation of succinic acid from glutamic acid that β-hydroxyglutamic acid might by analogy furnish malic acid.

$$COOH\cdot CH\cdot NH_2\cdot CH_2\cdot CH_2\cdot COOH \longrightarrow COOH\cdot CH_2\cdot CH_2\cdot COOH$$

$$COOH\cdot CH\cdot NH_2\cdot CHOH\cdot CH_2\cdot COOH \longrightarrow COOH\cdot CHOH\cdot CH_2\cdot COOH$$

It was soon established that, while *l*-malic acid was undoubtedly formed in the experiments with β-hydroxyglutamic acid, corresponding blank experiments without the amino acid usually furnished as much malic acid and not infrequently even more. In the absence of evidence as to the direct origin of *l*-malic acid from β-hydroxyglutamic acid through the action of fermenting yeast, it was decided to examine other amino acids and related substances as possible precursors of malic acid. From a struc-

tural point of view ornithine or arginine, proline, hydroxyproline, glutamic acid, aspartic acid, and asparagine seemed the most likely, but in each case addition of these substances to the fermenting mixture resulted in a definite lowering of the malic acid production. Most other amino acids had a similar effect and none produced an increase. It seemed probable that the addition of these amino acids exercised a protein-sparing effect on the yeast protoplasm, resulting in a lowered malic acid production.

Asssuming that the malic acid originates from the yeast and not from the sugar—and there is fair evidence to support this view—it was of interest to separate the amino acids derived from the hydrolysis of yeast protein and examine them as to possible precursors of malic acid. Accordingly yeast protein was hydrolyzed and the amino acids were resolved into the following groups: (*a*) alcohol-soluble substances, chiefly proline, (*b*) monaminocarboxylic acids, extracted by butyl alcohol, (*c*) the strong bases, precipitated by phosphotungstic acid, (*d*) the dicarboxylic monamino acids, and (*e*) a residue containing neither strong acids nor bases. Of these, the first three classes and the last caused a pronounced lowering of malic acid formation, while the dicarboxylic acids produced but little change in either direction. There is therefore no reason to believe at present that some hitherto unknown amino acid occurring in yeast protein is the precursor of fermentation malic acid.

A similar reduction in malic acid production was observed when anti- or para-hydroxyaspartic acid, asparagine, or ammonium salts were added to the fermenting medium. The fact that an adequate source of nitrogen in the medium almost invariably results in diminished malic acid formation must be regarded as fairly strong evidence as to the probable formation of malic acid as the result of protein catabolism. The results in this respect are strikingly similar to those obtained from the study of succinic acid production by yeast.

In addition to the amino acids whose influence on malic acid production was studied, experiments were made with succinic, fumaric, and optically inactive malic acid. A definite and quantitatively considerable conversion of fumaric acid into *l*-malic acid was observed, but the other substances were almost without effect. It is an open question as to whether fumaric acid

is concerned with the formation of fermentation malic acid, although the production of the former acid by moulds has been observed by Ehrlich (2). In Ehrlich's experiments it was fairly conclusively shown that the fumaric acid originated from carbohydrate so that the analogy with the present results is not a close one. Succinic acid is surely not a precursor of malic acid under the prevailing experimental conditions.

While traces of *l*-malic acid are to be found in stale or autolyzed yeast, its production is essentially bound up with the stimulated metabolism associated with active alcoholic fermentation. Within certain narrow limits, increase in sugar fermented with a fixed amount of yeast usually leads to additional malic acid.

One other point remains to be referred to; namely, the influence of the reaction of the medium on malic acid production. High acidity, whether induced by the addition of acids or by their formation by fermentation, is generally associated with low malic acid production. In making comparative tests the addition of a moderate amount of sodium bicarbonate or other mild alkali appears to have a distinctly favorable action on malic acid formation.

EXPERIMENTAL.

Unless otherwise stated the usual proportions adopted for the various fermentation experiments were as follows: pressed yeast or its equivalent 50 gm., cane-sugar 200 gm., sterilized tap water 1,500 cc., added substance 2.5 to 5 gm. In cases where sodium bicarbonate was added the amount varied from 2 to 5 gm.

The yeast was of various sources, most of the experiments being made with a specially pure starch-free commercial preparation or with pure yeast grown in malt extract with close bacteriological control. For generous supplies of yeast of various descriptions the writer is indebted to The Fleischmann Company and to the courtesy of S. R. E. Lee. Considerable variations in the ability of different samples of pure yeast to produce malic acid were noted and in the event of heavy bacterial contamination its formation was always diminished. Some degree of nitrogen starvation appeared to favor the production of malic acid.

Fermentation was conducted in large flasks loosely plugged with cotton and was continued until all sugar had disappeared, the

time required varying from 1 to 5 days. The flasks were occasionally shaken, but no particular effort was made either to avoid or promote absorption of oxygen. At the close of the fermentation the yeast was removed by filtration through a V grade Berkefeld filter. This was found superior to the Pukal flask or other similar devices.

Isolation of l-Malic Acid Produced during Fermentation.—The following method permitted the ready isolation and identification of *l*-malic acid. Filtrates from fermentations conducted as above described, in which nothing but pure yeast and sterile cane-sugar solution had been used, were concentrated under reduced pressure to a thin syrup. It was then made feebly alkaline with ammonia and filtered. Powdered lead acetate was then added in excess and precipitation continued with hot basic lead acetate solution until further addition produced no further precipitation. An equal volume of alcohol was then added and the mixture allowed to stand overnight. The precipitate was then filtered off, washed, suspended in water, and then decomposed with hydrogen sulfide. The filtrate was concentrated to small bulk and allowed to crystallize so as to remove the bulk of the succinic acid. The syrupy mother liquor was next thoroughly extracted with ether. The ether extract was almost entirely a mixture of malic and succinic acids. It was dissolved in water and its acidity determined by titrating a small part. The remainder was evaporated to dryness and dissolved in 10 parts of acetone. An amount of powdered cinchonine barely sufficient to form the acid salt of the dibasic acid, was then added to the hot solution. Under these conditions cinchonine succinate, which is extremely soluble in acetone, remains in solution, while the very sparingly soluble cinchonine malate crystallizes very readily. The cinchonine malate was filtered off, washed with acetone, and then recrystallized from water. The properties of the salt were identical in every particular with those of cinchonine *l*-malate recently described by the writer (3). It melted at 197–198° (uncorrected), and the melting point was unchanged on mixing it with cinchonine *l*-malate of other origin. The yield of pure cinchonine *l*-malate in most experiments was from 1 to 3 gm. per liter of the fermented liquid, equivalent to about one-fourth of this amount in terms of malic acid.

About 30 gm. of pure cinchonine *l*-malate were thus obtained without difficulty and most of this was converted into free malic acid. The salt was dissolved in water, the alkaloid precipitated with ammonia, and the filtrate concentrated to a syrup which was acidified with phosphoric acid and extracted with ether for 24 hours. The ether residue was dissolved in a little hot water and allowed to crystallize. The malic acid melted at 99–100° and gave satisfactory results on combustion.

Found.	C 35.6, H 4.52.
Calculated.	" 35.8, " 4.48.

The specific rotation in water was −2.31° in 7 per cent aqueous solution while the rotations in the presence of uranium acetate and ammonium molybdate were −485° and +695° under the conditions specified in the paper previously referred to. The complete chemical and optical identity of the product with ordinary *l*-malic acid was thus established.

Effect of Amino Acids and Other Substances on Malic Acid Formation.—The conditions of fermentation were those previously outlined. In every case at least one blank experiment was run under identical conditions. This is essential since considerable variations are noted in the amounts of malic acid according to the condition of the yeast. At the close of fermentation, the liquid, filtered through a Berkefeld V filter, was concentrated and precipitated with neutral and basic lead acetates as described in the preceding section. The lead precipitate was then decomposed with hydrogen sulfide and the filtrate made up to a known small volume. An estimation of the amount of malic acid was made by polarizing the solution in the presence of uranium acetate under the conditions previously defined. Special experiments showed that no other substance than malic acid was present, which produced the enormous increase in optical rotation characteristic of uranium malate. In many cases readings were also made with the addition of ammonium molybdate, the rotation in this case being changed in sign. In all cases the solutions were examined polarimetrically before adding uranium acetate and showed uniformly a very slight levo rotation. This method is by no means strictly quantitative as the lead precipitation is apt to be imperfect even when alcohol is used. But the results

TABLE I.

Experiment.	Added substance.	Per liter.	$NaHCO_3$ per liter.	*l*-Malic acid found per liter.
		gm.	*gm.*	*mg.*
I	Blank.			72
	"		3.3	203–246
	Na β-hydroxyglutamate.	2.0	3.3	271–289
	" glutamate.	3.0	3.3	40
II	Blank.			51
	"		3.0	190–211
	Na β-hydroxyglutamate.	2.0	3.0	181–216–251
III	Blank.		3.0	122
	Na β-hydroxyglutamate.	3.0	3.0	69–75
	" aspartate.	3.0	3.0	21
	" hydroxyaspartate (anti).	3.0	3.0	11
	" " (para).	3.0	3.0	11
IV	Blank.			518
	Glutamic acid.	3.0		45
	Hydroxyglutamic acid.	3.0		59
	Proline.	3.0		225
V	Blank.			799–820
	Ammonium carbonate.	2.0		183–201
	Aspartic acid.	3.0		220
	Asparagine.	2.0		53–95
	Hydroxyproline.	2.0		505–717
VI	Blank.			62–81
	"		3.0	210–320
	Proline.	3.0	3.0	28–90
	Hydroxyproline.	3.0		123–187
VII	Blank.		3.0	461
	Monamino acids from yeast.	3.0	3.0	102
	Alcohol-soluble acids from yeast.	3.0	3.0	210
	Diamino acids from yeast.	3.0	3.0	64
	Dicarboxylic acids from yeast.	3.0	3.0	407
VIII	Blank.			352
	Fumaric acid.	2.0		680
	m-Malic "	2.0		229
	Succinic "	2.0		360
IX	Blank.		3.3	609
	Na fumarate.	3.3	3.3	1,020
	" *m*-malate.	3.3	3.3	702
	" succinic.	3.3	3.3	678

are at least comparable and in each case the figures are to be regarded as minimum values. The malic acid is expressed in terms of milligrams per liter of fermented liquid.

Table I contains the principal results.

The results do not require much detailed analysis. It is clear that great variations occur in malic acid production and that the addition of sodium bicarbonate to the fermenting fluid by preventing accumulation of free acid exerts a very favorable influence. Ammonium salts, monamino, glutamic, aspartic, and hydroxyaspartic acids definitely reduce malic acid production, while hexone bases lower it to almost the vanishing point. The action of proline and hydroxyproline is less marked, but on the whole unfavorable. Hydroxyglutamic acid in the presence of sodium bicarbonate sometimes gives a slight increase in malic acid, but its action is not regular and definite conclusions are unwarranted. Sodium fumarate gives a marked increase, while succinic acid produces little change.

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THE ACID-BASE EQUILIBRIUM OF THE BLOOD OF NORMAL GUINEA PIGS, RABBITS, AND RATS.

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Accurate studies in the acid-base equilibrium of the blood have been confined almost entirely to man and the larger laboratory animals. In order to make it possible to utilize the smaller animals a method was devised by means of which the determinations could be made with a very small amount of blood (1). With this method at hand a survey has been made of the normal variation in the blood of the three most used laboratory animals; namely, rabbits, guinea pigs, and rats.

Acid-Base Equilibrium of Guinea Pigs.

Guinea pigs were kept for several days under uniform conditions on a regular diet. 12 hours before the observations were made all food scraps were removed from the cages, and during all the manipulation the animals were handled carefully so as to avoid exciting them. The blood was drawn directly from the heart by attaching a needle with a short rubber tube to a 1 cc. pipette graduated to hundredths. 0.25 cc. of the whole blood was immediately transferred under oil to a saline indicator tube, thoroughly mixed, and then centrifuged. The pH values were then read in a comparator block at 20°C. and a constant subtracted to obtain absolute pH values at 38°C.[1] The pH of the whole blood was determined by Cullen's colorimetric method as modified by Hawkins (1), and the Van Slyke method (2) was used for determining the carbon dioxide content. The CO_2 content of the whole blood was determined before the blood clotted so that it was not necessary to coat the pipette with potassium oxalate.

[1] The method for determining the constants will be published in a subsequent paper.

TABLE I.

Guinea Pigs.

No.	pH	CO_2	No.	pH	CO_2
		Arterial blood.			
1	7.52	38.7	7	7.26	38.4
2	7.36	38.8	8	7.38	40.1
3	7.40	29.5	9	7.32	30.7
4	7.56	31.2	10	7.35	47.6
5	7.20	46.6	11	7.18	40.4
6	7.30	47.2	12	7.34	46.7
		Venous blood.			
1	7.27	38.0	6	7.16	57.9
2	7.35	45.6	7	7.21	39.3
3	7.19	40.6	8	7.29	48.0
4	7.12	39.9	9	7.26	51.4
5	7.16	41.0	10	7.32	51.2

TABLE II.

Guinea Pigs.

No.	0 hrs.		48 hrs.		10 days.	
	pH	CO_2	pH	CO_2	pH	CO_2
			Arterial blood.			
1	7.56	31.2	7.43	34.6		
2	7.38	45.8	7.50	43.7		
3	7.39	45.5	7.38	47.2		
4	7.42	41.4	7.45	42.6		
			Venous blood.			
1	7.16	57.9	7.35	54.2		
2	7.27	38.0	7.24	45.2	7.33	48.9
3	7.26	38.8	7.33	52.1		
4	7.35	45.6	7.45	52.7		
5	7.26	38.5	7.32	55.6	7.34	50.4
6	7.22	43.6	7.32	48.9	7.35	53.4

In determinations on the arterial blood of twelve individual guinea pigs, the pH values varied from 7.18 to 7.56 and the CO_2 content from 29 to 47 volumes per cent (Table I and Fig. 1). Not only is there a great variation between different guinea pigs,

but determinations made at 48 hour intervals on the same animal show a very marked fluctuation (Table II).

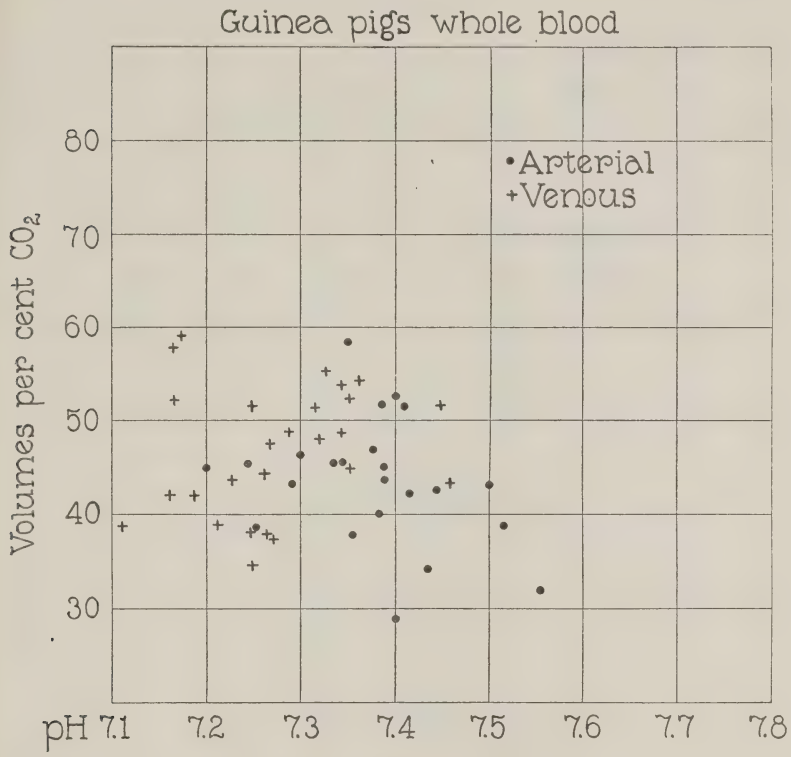

Fig. 1.

Acid-Base Equilibrium of Rabbits.

Using the same technique as that described above, determinations were made on the arterial blood of forty-four normal rabbits and the venous blood of ten rabbits. In these animals the pH varied from 7.26 to 7.58 in the arterial blood and from 7.24 to 7.45 in the venous. These figures fall within the range of the figures published by Hasselbalch and Lundsgaard (3) (7.15 to 7.45), Menten and Crile (4) (7.18 to 7.70), Hussey (5) (7.12 to 7.26), and Kurijama (6) (7.35 to 7.50).

TABLE III.

Rabbits.

No.	pH	CO_2	No.	pH	CO_2	No.	pH	CO_2
				Arterial blood.				
1	7.46	62.0	16	7.34	44.6	31	7.49	42.3
2	7.47	50.8	17	7.30	31.8	32	7.40	41.0
3	7.41	50.1	18	7.35	42.4	33	7.41	37.6
4	7.55	62.1	19	7.23	44.2	34	7.50	44.4
5	7.44	29.4	20	7.39	35.6	35	7.50	44.2
6	7.59	42.6	21	7.41	44.8	36	7.56	43.2
7	7.47	44.6	22	7.41	51.3	37	7.37	40.6
8	7.37	32.6	23	7.31	28.8	38	7.46	49.3
9	7.38	37.3	24	7.51	49.5	39	7.31	41.9
10	7.42	38.8	25	7.55	60.5	40	7.27	52.6
11	7.38	38.9	26	7.34	43.7	41	7.30	48.3
12	7.39	77.0	27	7.29	29.6	42	7.57	42.6
13	7.45	52.6	28	7.34	34.6	43	7.31	26.4
14	7.46	48.7	29	7.26	23.9	44	7.57	49.5
15	7.47	39.3	30	7.40	34.6			
				Venous blood.				
1	7.31	38.8	6	7.48	62.1			
2	7.40	73.3	7	7.38	59.1			
3	7.31	49.3	8	7.27	55.0			
4	7.26	45.7	9	7.37	33.5			
5	7.32	51.4	10	7.32	46.0			

TABLE IV.

Mixed Blood of Rats.

No.	pH	CO_2	No.	pH	CO_2	No.	pH	CO_2
1	7.40	62.1	15	7.52	59.2	29	7.49	62.1
2	7.42	64.5	16	7.43	57.1	30	7.46	59.7
3	7.40	44.7	17	7.42	50.6	31	7.43	58.6
4	7.49	58.3	18	7.53	57.5	32	7.50	64.0
5	7.40	48.2	19	7.50	55.0	33	7.52	56.2
6	7.35	54.6	20	7.51	60.6	34	7.51	56.3
7	7.36	59.2	21	7.48	60.7	35	7.48	61.6
8	7.41	62.6	22	7.46	57.6	36	7.46	58.7
9	7.35	47.2	23	7.50	57.2	37	7.53	50.6
10	7.38	65.0	24	7.55	55.0	38	7.52	53.8
11	7.47	61.2	25	7.47	52.4	39	7.52	53.6
12	7.51	49.7	26	7.45	53.1	40	7.50	53.2
13	7.50	61.5	27	7.47	53.7			
14	7.53	50.6	28	7.49	59.7			

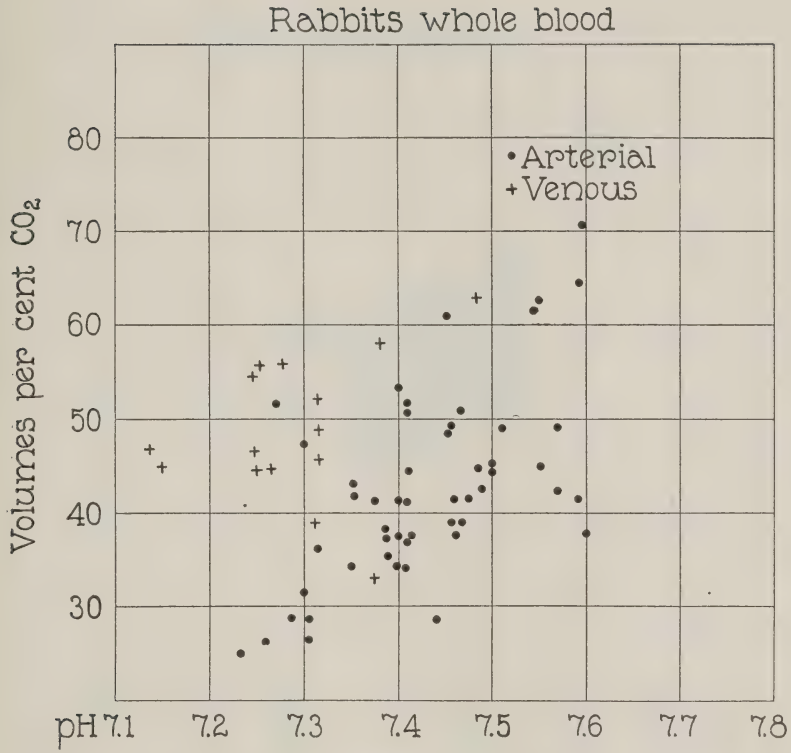
Rabbits whole blood
•Arterial
+Venous
Volumes per cent CO_2
80
70
60
50
40
30
pH 7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8

Fig. 2.

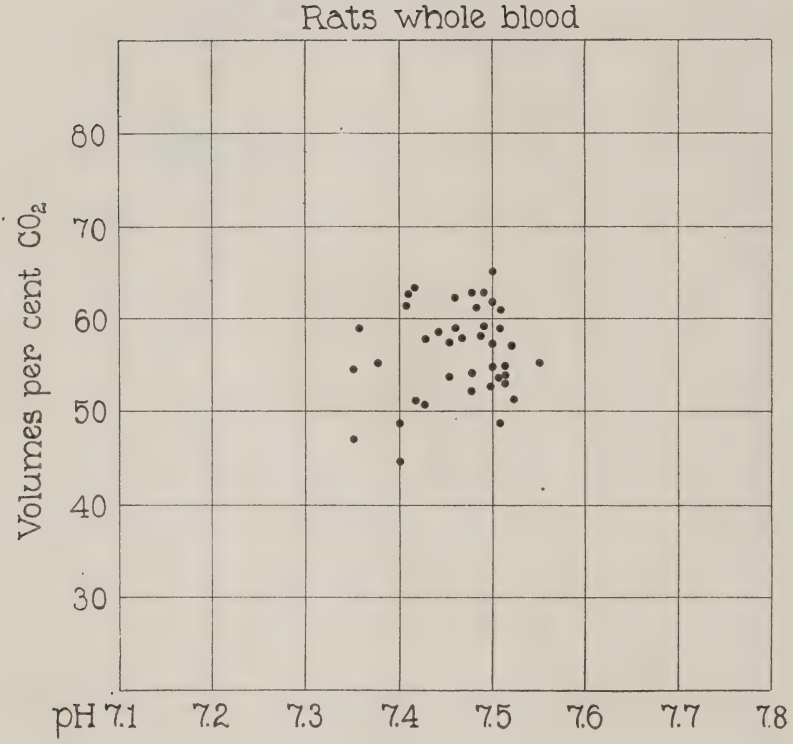
Rats whole blood
Volumes per cent CO_2
80
70
60
50
40
30
pH 7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8

Fig. 3.

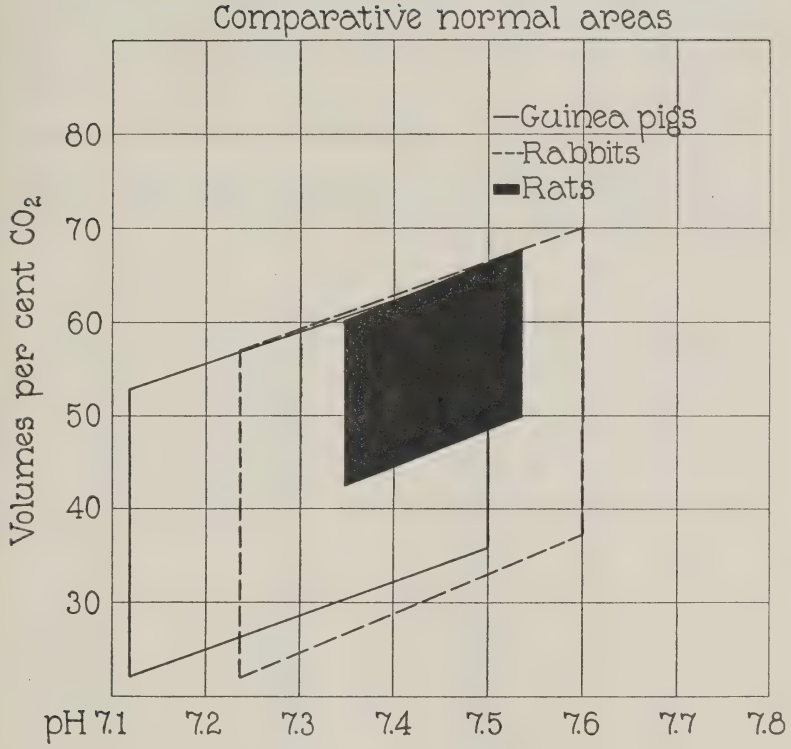
Comparative normal areas
—Guinea pigs
---Rabbits
Rats
Volumes per cent CO_2
80
70
60
50
40
30
pH 7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8

Fig. 4.

The CO_2 determination of the arterial blood of rabbits varied from 25 to 54 volumes per cent and that of the venous blood from 39 to 61 volumes per cent (Table III and Fig. 2).

Reaction of the Blood of Normal Rats.

Blood from rats was obtained by decapitating the animals and allowing the blood to drip under oil into a small glass cone. The determinations were then made by the methods already described. In forty rats studied the pH value of the whole blood varied from 7.36 to 7.55 and the CO_2 from 44 to 60 volumes per cent (Table IV and Fig. 3).

For comparison the range of variation for guinea pigs, rabbits, and rats is plotted in Fig. 4.

TABLE V.

Temperatures in Degrees Centigrade.

No.	Rats.	Rabbits.	Guinea pigs.
1	38.0	38.8	37.0
2	37.9	39.6	38.2
3	38.0	38.9	39.1
4	38.0	38.9	37.5
5	38.0	39.6	39.3
6	38.0	39.3	39.3
7	38.2	38.2	37.4
8	38.1	39.6	38.3
9	38.0	39.3	38.0
10	38.4	39.0	38.2
11	38.2	39.1	38.7
12	38.3	39.2	39.4
13	38.2	39.3	39.1
14	38.5	39.2	38.4
15	38.6	39.3	37.8
16	38.0	39.3	39.4
17	38.7	39.2	39.3
18	38.2	39.1	39.0
19	38.2	38.9	39.4
20	38.2	38.9	39.6
Maximum variation	0.8°	1.4°	2.6°

Body Temperature Variations.

An observation which may or may not be of importance in this connection is the range of normal variation in body temperature (rectal) in the three types of animal used in these experiments when kept under normal laboratory conditions. The maximum variation in the rats was only 0.8°C., while the rabbits varied as much as 1.4°C., and the guinea pigs, 2.6°C. (Table V).

DISCUSSION.

From these observations it is apparent that rabbits and guinea pigs are not very satisfactory animals for experiments involving the study of the acid-base equilibrium. They not only show a great individual variation, but the same individual varies from day to day. On the other hand, the rat shows considerable constancy both in the pH and CO_2 content of the whole blood. It is interesting to note that the guinea pig which shows the greatest variations in the acid-base equilibrium also has the least stable body temperature, while the rat with a relatively stable body temperature has the smallest fluctuation in the reaction of the blood. In both respects the rabbit falls between the two.

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ON LIGNOCERIC ACID.

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(Received for publication, June 16, 1924.)

Lignoceric acid has been isolated by several careful workers and, invariably, every one recorded for it a melting point of 80–81°C. On the basis of this finding, Meyer, Brod, and Soyka recognized that lignoceric acid had a structure different from normal tetracosanic acid which melted at 85°C. The conclusions of Meyer, Brod, and Soyka[1] were substantiated by Levene and West.[2] In 1921, there appeared a publication by Brigl and Fuchs[3] in which all the past work on lignoceric acid was assailed and the previously reported melting point was declared erroneous. That which previous workers had described under the name of lignoceric acid, according to Brigl and Fuchs, is a mixture of normal tetracosanic acid melting at 85°C., and a second tetracosanic acid melting at 75°C. The separation of the low melting from the high melting acid was accomplished through crystallization from pyridine. The higher melting material was then recrystallized successively from benzene, alcohol, and toluene. Thus, the procedure seems very simple and one wonders how every previous worker overlooked the point emphasized by Brigl and Fuchs.

Lignoceric acid is a constituent of cerebrosides and of sphingomyelin and because of this, we were always interested in its structure. It was natural, therefore, that we should have undertaken to test the correctness of the conclusions of Brigl and Fuchs. In the course of the work on the higher fatty acids and on the higher hydrocarbons, we often had occasion to observe that on standing for several years in sample bottles, the substances melted at lower

[1] Meyer, H., Brod, L., and Soyka, W., *Monatsh. Chem.*, 1913, xxxiv, 1113.

[2] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1914, xviii, 477.

[3] Brigl, P., and Fuchs, E., *Z. physiol. Chem.*, 1922, cxix, 280.

temperatures than they did when freshly prepared. Since all our own data on the physical properties of lignoceric acid were obtained on *fresh materials,* we concluded to test the Brigl and Fuchs procedure for the purification of the acids freshly prepared from two sources; namely, from peanut oil and from cerasin. Each of these acids was recrystallized from pyridine and, subsequently, from the other solvents, following the directions of Brigl and Fuchs. The two substances prepared from different sources had exactly the same melting point of 80.5–81°C. and a mixture of the two substances also melted at the same temperature.

Thus, it seems there is no reason to revise our older conclusions that lignoceric acid is not identical with the normal tetracosanic acid. It is possible that beechwood tar is not a satisfactory material for the preparation of unaltered lignoceric acid.

Lignoceric Acid from Peanut Oil.

The water-insoluble fatty acids from the saponification of 60 pounds of peanut oil served as the source of lignoceric acid. The mixture of acids at ordinary room temperature is semiliquid and was separated into liquid and solid fractions in the centrifuge. The solid fraction was then suspended in 95 per cent alcohol and again separated by centrifugalization. Repetition of this washing with 95 per cent alcohol removed most of the adhering liquid fatty acids. The solid mixture was crystallized from 95 per cent alcohol at room temperature, after which it melted at 73.5–74.5°C. and weighed 500 gm. Further crystallization from alcohol failed to raise the melting point more than 0.5°C. Solid acids that could be separated from the alcohol used for washing and crystallizing apparently contained little lignoceric acid and were discarded.

The top fraction was crystallized three times from pyridine (88 cc. per gm.) at 7–8°C., melted over dilute hydrochloric acid on the water bath, and crystallized from acetone. It then melted at 78.5–79.5°C. Four crystallizations of this product from ether (10 to 15 cc. per gm.) at 7–8°C. raised the melting point to 80–81°C. It solidified sharply at 77.5°C. It was then analytically pure and on titration in hot benzene-methyl alcohol solution with 0.1 N NaOH the correct figure was obtained for the acid number (calculated as molecular weight).

0.1005 gm. substance: 0.2878 gm. CO_2 and 0.1173 gm. H_2O.
0.7424 " " required 19.98 cc. 0.1 N NaOH.
$C_{24}H_{48}O_2$. Calculated. C 78.15, H 13.13, Mol. wt. 368.
Found. " 78.09, " 13.06, " " 371.5.

The lignoceric acid was now fractionated in exactly the manner described by Brigl and Fuchs for the acid they obtained from beechwood tar. It was crystallized from ether (20 cc. per gm.) at 9–10°C., three times, without any change in the melting or solidification point. The crystallization was repeated using benzene as solvent (15 cc. per gm.) four times, with absolute ethyl alcohol (15 cc. per gm.) three times, and once with toluene (15 cc. per gm.). The temperature in each case was 9–10°C. Although 20 per cent of the material remained in the mother liquors, the melting point remained constant at 80–81°C.

The acid was then crystallized from pyridine (8 cc. per gm.) at 10°C., four times. The loss in the mother liquor was 40 per cent of the sample taken. The least soluble fraction again melted at 80–81°C. and solidified sharply at 77.5°C.

0.1002 gm. substance: 0.2880 gm. CO_2 and 0.1185 gm. H_2O.
0.7350 " " required 19.80 cc. 0.1 N NaOH.
$C_{24}H_{48}O_2$. Calculated. C 78.15, H 13.13, Mol. wt. 368.
Found. " 78.38, " 13.24, " " 371.2.

Finally, the acid was converted into the ethyl ester. It was dissolved in 99.5 per cent ethyl alcohol, sulfuric acid added, and the solution boiled overnight. The ester was separated at 0°C. and the treatment repeated. After crystallization from acetone it melted at 56–57°C. and solidified at 54.5°C. It distilled completely at 220–222°C. at 0.50 mm. with the bath at 260°C. and then melted at 55.5–56.5°C. and solidified at 55–54.5°C.

The ethyl lignocerate was saponified with alcoholic sodium hydroxide; the salt precipitated with acetone and washed with water. It was decomposed over dilute hydrochloric acid on the water bath; the acid crystallized from acetone and passed over the lead salt. The melting point remained constant at 80–81°C. It also solidified sharply at 77.5°C.

The melting points are corrected.

Lignoceric Acid from Cerasin.

This lignoceric acid was obtained from the material more soluble in methyl alcohol on fractionation of mixtures of cerebrosides isolated from brains of cattle. The rotation of the cerebroside was determined in pyridine.

$$[\alpha]_D^{22} = \frac{+0.07^\circ \times 100}{0.5 \times 8.0} = +1.75^\circ$$

800 gm. of cerebrosides were hydrolyzed in 200 gm. lots from which 60 gm. of impure lignoceric acid were obtained. The cerebroside (200 gm.) was dissolved in 2,000 cc. of absolute ethyl alcohol containing 140 gm. of concentrated sulfuric acid and boiled under a reflux condenser on the water bath for 7 hours. The ester which crystallized at room temperature (20°C.) was filtered off. It was dissolved in 10 parts of absolute ethyl alcohol containing 2 per cent sulfuric acid and again boiled under a reflux condenser on a water bath for 5 hours. The ester which crystallized at room temperature was filtered off and crystallized from acetone. It melted at 56°C. (corrected). The ester was converted to the soap with sodium hydroxide in the usual way and the soap decomposed with dilute hydrochloric acid in the usual manner. The free acid (No. 109) had a melting point of 79°C. (corrected).

A portion of the free acid (No. 109) was recrystallized three times from pyridine and the pyridine removed with acetone. The melting point remained unchanged. It was then converted to the lead salt and after decomposing this, the free acid was recrystallized four times from toluene. It then melted at 80°C. Three recrystallizations from absolute ethyl alcohol did not change the melting point. The acid was then recrystallized four times from chloroform and finally from acetone. It melted at 81°C. (corrected), and solidified at 77.5°C. (corrected).

0.1000 gm. substance: 0.2858 gm. CO_2 and 0.1182 gm. H_2O.
0.6884 " " required 18.60 cc. 0.1 N NaOH.
$C_{24}H_{48}O_2$. Calculated. C 78.15, H 13.13, Mol. wt. 368.
Found. " 77.94, " 13.26, " " 370.

Another portion of the free acid (No. 109) was esterified with absolute ethyl alcohol containing 3 per cent sulfuric acid. The

ester which crystallized at room temperature was filtered off and crystallized from acetone. It melted at 56°C. (corrected). The ester was then distilled *in vacuo* into two fractions. The fraction which distilled over first (top fraction) was saponified and converted to the free acid in the usual manner. The acid was recrystallized three times from chloroform and finally from acetone. It melted at 81°C. (corrected). Three recrystallizations from pyridine and three recrystallizations from absolute ethyl alcohol did not change the melting point.

0.0992 gm. substance: 0.2850 gm. CO_2 and 0.1172 gm. H_2O.
0.6954 " " required 18.70 cc. 0.1 N NaOH.
$C_{24}H_{48}O_2$. Calculated. C 78.15, H 13.13, Mol. wt. 368.
Found. " 78.34, " 13.22, " " 372.

THE FORMATION OF *l*-LEUCIC ACID IN THE ACETONE-BUTYL ALCOHOL FERMENTATION.*

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Plate 1.

(Received for publication, June 23, 1924.)

In the fermentation of sugars, starch, and other carbohydrates by *Bacillus granulobacter pectinovorum*, the chief products are butyl alcohol, acetone, ethyl alcohol, and acetic and butyric acids. Reilly and his associates (1) pointed out in 1920 that the fermented media contained a non-volatile acid which increased in amount with the age of the culture. The quinine salt prepared from the calcium salt of this non-volatile acid, on hydrolysis with baryta, gave a mixture of barium salts which pointed to the existence of another acid with an equivalent equal to or greater than that of butyric acid. Recently, Speakman (2) has concluded, on the basis of qualitative tests, that the unknown acid is lactic acid, and on this conclusion, has constructed a theory for the fermentation of various sugars. This conclusion is based on doubtful evidence as another acid present in granulobacter fermentations gives the tests which he attributes to lactic acid.

In the fall of 1922 we obtained the zinc salt of a non-volatile acid which gave all the qualitative tests mentioned by Speakman, but whose zinc content and water of crystallization did not agree with that of zinc lactate. In addition to this quantitative data, certain physical properties, such as manner of crystallizing and decrease in solubility of the zinc salt on heating were different from those of zinc lactate.

*This work was supported in part by a grant from the special research fund of the University of Wisconsin. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

In the experimental part of this paper, we shall present evidence to show that the non-volatile acidity is due in part at least to the presence of *l*-leucic acid (α-hydroxyisocaproic acid).

EXPERIMENTAL.

6 per cent corn mash (30 liters) was sterilized and inoculated with a vigorously fermenting culture of *B. granulobacter pectinovorum*. Several days after gassing had ceased, the mash was filtered through cotton and cheese-cloth and the greenish yellow filtrate was placed in large pans and evaporated on the steam bath at a temperature below 70°C. with the aid of a fan to a volume of about 800 cc. This thick dark liquid was acidified with H_2SO_4 and placed in Kutcher-Steudel extractors and extracted with ether for several days. The ether extracted a light yellow pigment as well as the acids. The ether was removed from the receiver and the extract subjected to steam distillation to remove volatile acids.

The acid solution was first boiled with animal charcoal to decolorize the solution, but some of the yellow pigment still remained after this treatment. Attempts were made to prepare a number of salts by neutralizing the acid with the hydroxide or carbonate of the metal, and evaporating the solutions. The potassium and ammonium salts proved to be syrupy, with no tendency to crystallize. The lead, cadmium, and barium salts showed some crystalline structure, but not to the same degree as the calcium and zinc salts. The preparation and analysis of these two salts only, are given.

Calcium Salt.

A part of the acid solution was neutralized with lime water and slowly evaporated at 65°C. A scum of colorless crystals, somewhat needle-like, formed on the surface, but their crystalline structure was not very definite. The salt was filtered off, washed with water, dried to constant weight at 105°C., and analyzed with the following result:

0.0742 gm. salt : 0.0142 gm. CaO.
Ca in CaO equivalent to 9.6 cc. 0.05 N $KMnO_4$.
Percentage Ca in salt : 13.7, 12.9.
Theory for $(C_6H_{11}O_3)_2Ca$: 13.25.

Zinc Salt.

The acid solution was neutralized with Ba $(OH)_2$, and the barium precipitated with an equivalent quantity of 0.2 N $ZnSO_4$. The filtrate from the $BaSO_4$ was evaporated on the steam bath. Large, dirty white scales or flakes immediately began to form on the surface of the liquid. When evaporated to a small volume this scaly mass was filtered, washed with water, and redissolved in a large volume of water. These scales or flakes went into solution with great reluctance as they seemed oily, and only slowly became wet when in contact with water. The dark impurities in the salt were slowly

eliminated by successive recrystallizations until finally about a gram of white feathery crystals were obtained. Under the microscope they looked much like match sticks, and were rhombic in form.[1]

Water of Crystallization of the Zinc Salt.—The zinc salt was recrystallized from water several times and then dried to constant weight in a desiccator

TABLE I.

Water of Crystallization Contained in the Zinc Salt.

No.	Weight of zinc salt.	Water lost.		Theory for $(C_6H_{11}O_3)_2Zn + 1.5\ H_2O$.
	gm.	*gm.*	*per cent*	*per cent*
1*	0.8662	0.0647	7.5	7.6
2*	0.2400	0.0170	7.0	7.6
3*	0.2642	0.0202	7.6	7.6
4*	0.5236	0.0382	7.3	7.6
5†	0.2000	0.0147	7.4	7.6

*Each analysis was made on a salt obtained from a different fermentation.

†Salt obtained from the mother liquors of No. 4.

TABLE II.

Zinc Content of the Salts.

No.*	Weight of zinc salt.	Weight of zinc oxide.	Percentage of zinc.	
			Found.	Theory for $(C_6H_{11}O_3)_2Zn$.
	gm.	*gm.*	*per cent*	*per cent*
1	0.0948	0.0226	19.2	19.96
2	0.0620	0.0148	19.2	19.96
3	0.1346	0.0334	19.9	19.96
4	0.0975	0.0234	19.3	19.96
5	0.0815	0.0200	19.7	19.96
6	0.0742	0.0182	19.7	19.96
7	0.0820	0.0206	20.0	19.96

*Each analysis was made on a salt obtained from a different fermentation.

[1] It was thought that the non-volatile acid might come from the fatty acids of the corn oil, so a quantity of fat-free corn-meal was fermented. After a number of recrystallizations about 0.25 gm. of the zinc salt was isolated, which had 7.6 per cent of water of crystallization and 19.4 per cent of zinc. These data show that the non-volatile acid is the same as that produced from the unextracted corn. The fats of the corn-meal are, therefore, not the source of the non-volatile acid.

TABLE III.

Combustion Data for the Zinc Salts.

Salt.	No.	Weight of sample.	Zinc.			Carbon.			Hydrogen.		
			ZnO	Found.	Theory.	CO_2	Found.	Theory.	H_2O	Found.	Theory.
		gm.	*gm.*	*per cent*	*per cent*	*gm.*	*per cent*	*per cent*	*gm.*	*per cent*	*per cent*
Zinc lactate	1	0.1990	0.0660	26.64	26.85	0.1990	27.30	29.50	0.0710	3.97	4.10
	2	0.2030				0.2220	29.83	29.50	0.0760	4.16	4.10
" hydroxyisocaproate	3	0.1346	0.0334	19.93	19.96	0.2180	44.15	43.97			6.60
	4	0.1252				0.2042	44.43	43.97	0.0751	6.66	6.60
	5	0.1230				0.2015	43.89	43.97	0.0725	6.55	6.60

over $CaCl_2$. The water of crystallization was determined by heating to 105°C. Heating the salt to a higher temperature, 130°C., did not drive off any more water, nor was any further elimination secured by heating for 2 hours at 135°C. and 12 mm. pressure. No indication of decomposition at these temperatures was shown by the salt. The data are given in Table I. The percentage obtained was 7.0 to 7.5, which is entirely different from that given by zinc lactate. The latter contains 12.9 and 18.2 per cent in the active and inactive salts, respectively.

Percentage of Zinc in the Salt.—The zinc content of a number of zinc salts secured from several different fermentations was determined by ignition. The data are given in Table II. The percentage of zinc is that required for $(C_6H_{11}O_3)_2Zn$.

Elementary Composition of the Zinc Salt.—A purified sample of the anhydrous salt was subjected to an ultimate analysis. After combustion, the boat was reweighed to secure the weight of residual ZnO. A sample of purified zinc lactate was first analyzed to see if a metallic salt could be successfully analyzed by combustion methods. The data are given in Table III, and show that the determination of carbon, hydrogen, and zinc agrees well with that required for $(C_6H_{11}O_3)_2Zn$.

Properties of the Zinc Salt.—The salt was insoluble in hot and cold chloroform, acetone, ether, glycerol, xylene, amyl alcohol, ethyl acetate, and methyl alcohol, and slightly soluble in water and water-alcohol mixtures.

100 cc. water (20°C.) dissolved 0.1810 gm.
Solubility—1:555 H_2O (20°C.).

In a recent paper Speakman (2) states that the non-volatile acid is lactic acid. From 5 liters of fermented corn mash he isolated a zinc salt which he reported "had the same structural characteristics as a sample of pure zinc lactate which was freshly prepared." The zinc salt which we have isolated in a similar manner also has the crystalline appearance of zinc lactate. Crystalline appearance is of little value in this case for the zinc salts of α-hydroxy fatty acids generally resemble each other in crystalline appearance (3). A microscopic examination of the zinc salt would not, therefore, yield conclusive evidence as to the identity of the acid. Speakman obtained "a positive result when small amounts of the material were submitted to the Uffelmann and the Hopkins-Cole tests." Mathews (4) states that the Uffelmann test is not specific for lactic acid, and we have found a number of organic acids which will respond positively to this test. Mathews also points out that the Hopkins test is not specific for lactic acid. Fletcher and Hopkins (5) assert that the production of the cherry-

red color is due to the reaction of the thiophene with the acetaldehyde which is formed by the action of the concentrated H_2SO_4 on the lactic acid. Malic acid also gave this test and it was assumed that the other α-hydroxy acids would respond to this test. Fearon (6) has shown that the Hopkins reaction for lactic acid is due to the production of formaldehyde and acetaldehyde, which then react with the thiophene. He found that the color varied slightly with different aldehydes—formaldehyde giving a purple-red, acetaldehyde a cherry-red, and acrolein a rose-carmine coloration. The test was also given with various substituted aldehydes. In addition to those named by Fearon, we have found that butyraldehyde, *n*-valeraldehyde, and isovaleraldehyde give a positive test.

A sample of our zinc salt in a 2 liter flask was dissolved in 300 cc. of water containing 2 cc. of concentrated H_2SO_4. When the solution was boiling vigorously 0.02 N $KMnO_4$ was run in as rapidly as the solution became decolorized. The distillate which was collected in an ice-cooled receiver had a sharp pungent odor, and was submitted to a number of aldehyde reactions. A portion of the distillate readily reduced ammoniacal silver nitrate, and turned a decolorized fuchsin solution pink, but did not react positively to the Lewin (7, 8) modification of the Rimini test which does not react with most aldehydes, but is very sensitive to acetaldehyde. A sample of freshly prepared zinc lactate responded positively to all three aldehyde reactions when oxidized in a similar manner. The evidence seemed conclusive that acetaldehyde was not contained in the distillate and hence the zinc salt could not be zinc lactate. Believing that a further study of this reducing substance would shed some light upon the nature of the acid, a number of aldehyde derivatives were prepared.

Preparation and Analysis of Various Aldehyde p-Nitrophenylhydrazones.

A sample of 0.25 gm. of the zinc salt was oxidized as above and the distillate collected in an ice-cooled receiver containing 0.4 gm. of *p*-nitrophenylhydrazine in 17 cc. of 50 per cent acetic acid. A curdy yellow precipitate composed of yellow needles soon formed in the receiver. The distillate was allowed to stand overnight in the ice box, and the precipitate filtered and dried at 65°C. Weight of the hydrazone, after several recrystallizations from 95 per cent alcohol, was 0.172 gm. and melted at

109–110°C. Under the same conditions, 0.25 gm. of zinc lactate yielded 0.154 gm. of acetaldehyde *p*-nitrophenylhydrazone which melted, after recrystallization from 95 per cent alcohol, at 128°C. The melting point given in the literature is 128°C.

The nitrogen content of these hydrazones was determined by the method of Milbauer (9). The hydrazone derived from the fermentation acid contained 19.3 per cent nitrogen, while that from the acetaldehyde gave 23.5 per cent, which is almost identical with the theoretical value. The nitrogen and melting point data clearly eliminate acetaldehyde, and point strongly to valeraldehyde as the oxidation product of the fermentation acid. However, there are three stereoisomeric valeraldehydes possible: normal valer-

TABLE IV.

Nitrogen Content and Melting Points of the p-Nitrophenylhydrazones of Three Aldehydes.

	p-Nitrophenylhydrazone of:		
	Acetaldehyde.	Isovaleral-dehyde.*	*n*-Valeral-dehyde.
Formula of hydrazone	$C_8H_9N_3O_2$	$C_{11}H_{16}N_3O_2$	$C_{11}H_{16}N_3O_2$
Nitrogen.			
Weight of sample, *gm*	0.0576	0.0542	0.0560
Acid neutralized, *cc.* N/14	13.53	10.47	10.44
Found, *per cent*	23.50	19.30	18.65
Theory, *per cent*	23.46	18.93	18.93
Melting point.			
Found, °*C*	128	110	75
Reported in literature, °*C*	128	110	?†

*By oxidation of the fermentation acid.

†Not found in any literature consulted.

aldehyde, isovaleraldehyde, and methylethyl acetaldehyde. The most practicable method for differentiating these aldehydes is by means of the melting points of their hydrazones. Dakin (10) and other investigators give the melting point of the hydrazone of isovaleraldehyde as 110°C. which is the same as that found for the unknown hydrazone. Neuberg and Peterson (11) found that the *p*-nitrophenylhydrazone of methylethly acetaldehyde melts at 112–113°C. A careful search of the literature failed to disclose any report on the preparation and melting point of the *p*-nitrophenylhydrazone of *n*-valeraldehyde.

A small quantity was therefore prepared by oxidizing a sample of *dl*-α-hydroxy-*n*-caproic acid which had been prepared from α-bromo-*n*-caproic acid. 10 gm. of α-bromo-*n*-caproic acid were hydrolyzed with aqueous KOH, acidifed with H_2SO_4, and extracted with ether. The ether was allowed to evaporate at room temperature in a crystallizing dish. The

α-hydroxy-*n*-caproic acid thus prepared had a great tendency to creep over the sides of the dish and crystallize on the outside. Alcohol was added to the oily product and allowed to evaporate at room temperature and then seeded with a few crystals obtained from the outside of the dish. When placed in the ice box, white crystalline flakes formed, which remained suspended in a yellow oil. When placed upon hardened filter paper, the residual oil was absorbed, leaving white flakes of the *dl*-α-hydroxy-*n*-caproic acid in a very pure condition. They had a pungent odor, a stringent taste, and melted sharply at 61–62°C. This checks very well with the results given by Abderhalden and Weil (12), Schulze and Likiernik (13), and Jelisafow (14, 15,). A sample was oxidized with permanganate in the usual manner. A curdy, yellow precipitate of the *p*-nitrophenylhydrazone formed in the cool receiver, and developed long straw-like needles upon standing in the ice box. After a number of recrystallizations from alcohol the melting point of the hydrazone remained at 74–75°C. It contained 18.65 per cent nitrogen. The low melting point of the hydrazone of *n*-valeraldehyde as compared with that of isovaleraldehyde is in agreement with what has already been observed in the case of the *p*-nitrophenylhydrazones of the butyraldehydes. These, according to Dakin (10), differ in their melting points by about 40°.

The data for the several aldehydes are collected in Table IV and show that the fermentation acid on oxidation with permanganate yields isovaleraldehyde. Additional evidence as to the nature of this aldehyde was obtained from the compound formed with dimethylhydroresorcinol.

Preparation of the Valeraldomedons.

The researches of Vorländer and Kalkow (16) have shown that aldehydes condense with dimethylhydroresorcinol, forming crystalline derivatives which can be readily purified, and have definite melting points within a convenient but well separated range. This condensation product can then be readily dehydrated by heating with acetic acid in a flask provided with a capillary stopper. The anhydrides also have characteristic melting points. This reagent does not react with ketones, hence it is possible to identify aldehydes in the presence of ketones. Neuberg and Reinfurth (17) have used this reagent to isolate acetaldehyde in alcoholic yeast fermentation, and Stepp and Feulgen (18) to identify acetaldehyde in diabetic urine. Neuberg has given this condensation product of acetaldehyde the abbreviated name of aldomedon, and the reagent the name of dimedon. Fricke (19)

has suggested that aldomedon be changed to acetaldomedon, and in this manner the "aldomedon" terminology can be applied to other aldehydes.

Using this reagent, a number of aldehyde derivatives were prepared in the following manner: 0.5 gm. of zinc salt was oxidized with $KMnO_4$ and the distillate (100 cc.) collected in a cooled receiver; 1.5 gm. of the dimedon in 18 cc. of 96 per cent alcohol were added to the distillate with stirring and then 3.6 gm. of NaCl. Upon standing, a heavy white crystalline precipitate formed, which was filtered off and dissolved in alcohol. Twenty times its volume of water was slowly added with vigorous stirring, whereupon the crystalline aldomedon separated out, but the unchanged dimedon remained in solution. After standing in the ice box, the precipitate was filtered and washed with water containing 5 per cent alcohol. Upon drying over P_2O_5 and recrystallizing from alcohol, the compound melted sharply at 154°C., which corresponds with what is given in the literature. Of the aldomedon 0.377 gm. was dehydrated by heating on the steam bath for 12 hours with 1.1 cc. of glacial acetic acid. Evaporation was limited by providing the flask with a stopper containing a capillary tube. Ten times its volume of water were then added and, upon standing in the ice box, a crystalline precipitate formed. The precipitate was filtered off and was digested on the filter several times with a 10 per cent solution of Na_2CO_3, and then washed with ice water. After recrystallization from alcohol, the anhydride melted at 167°C. A similar compound prepared from synthetic isovaleraldehyde was found to melt at 153°C. and the anhydride at 169°C. Stepp (8) gives 153–155°C. and 168°C., respectively, as the melting points of these compounds.

The literature contains a large number of references (20–28) demonstrating the fact that α-hydroxy acids are readily oxidized to the next lower aldehyde. Inasmuch as the aldehyde derived from the unknown zinc salt was found to be isovaleraldehyde, it is probable that the precursor of this aldehyde was α-hydroxyisocaproic acid.

Preparation of the α-Hydroxyisocaproic Acid.

In an effort to isolate the free acid, 1 gm. of the zinc salt was dissolved in a small amount of water containing sufficient H_2SO_4 to set free all the organic acid. The solution was placed in a Kutcher-Steudel apparatus and extracted continuously with ether for several days. The ether extract was then transferred to a crystallizing dish and the ether allowed to volatilize at room temperature. The oily residue had a tendency to creep over the sides and crystallize on the outside. Upon placing in the ice box and inoculating with a crystal secured from the outside of the dish, the entire

mass solidified, leaving a small amount of residual oil. This material was placed upon a hardened filter paper which absorbed most of the oil. Upon rapid recrystallization from alcohol, long feathery, fern-like streamers spread out in all directions as shown in Fig. 1, Plate 1. Upon very slow crystallization at ice box temperature, large rhomboid crystals, as shown in Fig. 2, Plate 1 were secured. These crystals were examined by Dr. A. N. Winchell of the Geology Department, University of Wisconsin, to whom we are indebted for the following description:

"Both are orthorhombic in crystallization, but decidedly different in crystalline habit. The first sample of crystalline acid is acicular, fibrous, probably elongated parallel to the vertical axis, and lying on one or other of the vertical pinacoids, but in most cases, on the brachypinacoid 0.010. The second set of crystals show well developed basal planes limited and modified by very low pyramids. Both kinds of crystals have parallel extinction, and correspond in all particulars with orthorhombic symmetry. The basal planes are bounded by prism faces, which show distinct curvature in some cases, and therefore the angle between the prism faces seems to vary. The value for the acute angle may be given as 72° (± 3°). The plane of the optic axis is parallel with the base, and the positive acute bisectrix is parallel with the base *b*. Therefore the sign of the elongation parallel to *c* is plus in some crystals and minus in other crystals."

The physical properties of these crystals agree with the description given by Haushofer (29) for *l*-leucic acid. The latter found the value of the acute angle to be 69.39°, but apparently did not observe that the prism faces possess a distinct curvature.

A sample of the fermentation acid which had been recrystallized from alcohol several times was found to melt at 75–77°C. When dissolved in 10 cc. of alcohol, 0.4690 gm. of the crystalline material showed a rotation of −2.50 on the Ventzke scale at 20°C., which is equivalent to a specific rotation of −9.23.

These results are in good agreement with what is given in the literature. α-hydroxy caproic acids have been frequently utilized as characterization compounds in the constitutional studies of the leucines. Certain properties of *l*-leucic acid as described by various observers, are tabulated in Table V. The melting points range from 70° to 82°C. with an average for thirteen determinations of 75°C. The specific rotation ranges from −4.14 to −16.37. Some of these determinations were made on such small quantities of acid that the observed rotation was too small to give accurate results. Three observers give figures in the neighborhood of −10.5 which is probably the correct value. A sample of *l*-leucic acid prepared from *l*-leucine had the same properties

as the fermentation acid. On the basis of all the evidence presented there can be no doubt that *Bacillus granulobacter pectinovorum* produces *l*-leucic acid as a normal fermentation product. It is also probable that in addition to leucic acid, other non-volatile acids are produced.

The source of the leucic acid cannot be stated at the present time, but the evidence strongly points toward protein. The main protein

TABLE V.

Properties of l-Leucic Acid.

Source.	Melting point.	$[\alpha]_D^{20}$	Year.	Observer.
	°C.			
Natural *l*-leucine.....	75		1848	Strecker (30).
" "	73		1861	Waage (31).
" "	73		1893	Schulze and Likiernik (13).
" " (casein)...	72.5	−4.4	1894	Gmelin (32).
" " (yeast)....	72.5	−4.57	1894	"
" " (hemoglobin).....	67–70	−10.5	1894	"
Natural *l*-leucine.....	78		1897	Röhmann (33).
" "	78–79		1910	Sachs (34).
" "	81–82	−10.4	1911	Scheibler and Wheeler (35).
" "	71	−16.37	1913	Abderhalden and Weil (12).
" "	74	−10.72	1921	Arai (36).
" "	77	−10.32	1921	"
Synthetic "	78–81	−10.4	1911	Scheibler and Wheeler (35).
Fermentation.....	75–77	−9.23	1924	Schmidt, Peterson, and Fred.

of corn, zein, contains 19.55 per cent of leucine (37). Unpublished data from this laboratory have shown that *Bacillus granulobacter pectinovorum* possesses great proteolytic powers. It is possible that the organism might readily deaminate the liberated leucine, with the production of leucic acid. In this connection, attention is called to the classical researches of Ehrlich and Pistschimuka (38) on the production of fusel oils from amino acids by the deaminating and decarboxylating action of yeast. A somewhat more closely

allied piece of work is that of Arai (36) on the production of *l*-leucic acid from *l*-leucine by *Proteus vulgaris*, and *d*-leucic acid from *l*-leucine by *Bacillus subtilis*. It is not improbable that the granulobacter organism acts in a similar manner.

SUMMARY.

1. A non-volatile acid produced by *Bacillus granulobacter pectinovorum* from corn mash has been isolated, and shown to be *l*-leucic acid (α-hydroxyisocaproic acid). The acid is orthorhombic in crystallization and the acute angle measures 72° (±3°). It has a melting point of 75–77°C. and a specific rotation of −9.23.

2. The acid yields a calcium salt corresponding to the formula $(C_6H_{11}O_3)_2Ca$. The zinc, carbon, hydrogen, and oxygen content of the zinc salt corresponds to that required for the salt, $(C_6H_{11}O_3)_2Zn$. The zinc salt crystallizes with 7 to 7.5 per cent of water ($1\frac{1}{2}$ H_2O).

3. Upon oxidation with $KMnO_4$, the acid yields an aldehyde which has been identified by the melting point and nitrogen content of its *p*-nitrophenylhydrazone, and the melting point of its aldomedon, as isovaleraldehyde.

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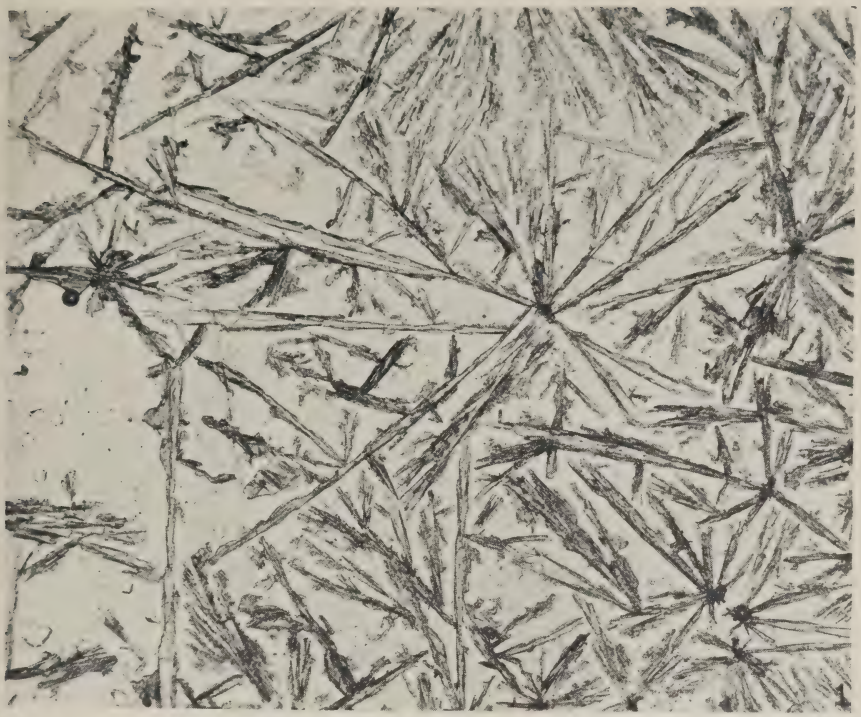
Addendum.—After this paper was in press a report by Sen, (Sen, H. K., *Biochem. Z.*, 1923, cxl, 447) was found in which the melting point of *p*-nitrophenylhydrazone of *n*-valer aldehyde is given as 74°C.

EXPLANATION OF PLATE 1.

FIG. 1. *l*-Leucic acid on rapid crystallization. × 4.
FIG. 2. *l*-Leucic acid on slow crystallization. × 4.



(Schmidt, Peterson, and Fred: Leucic acid by fermentation.)

A SIMPLIFIED METHOD FOR CHOLESTEROL DETERMINATION IN BLOOD.

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The method here described has the advantages over the older methods in that it is less time-consuming and is more accurate

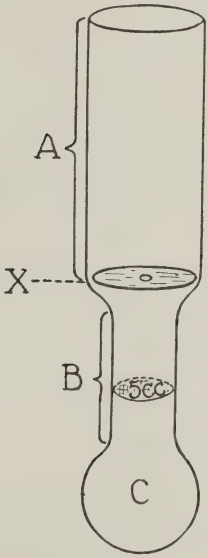

Fig. 1.

Fig. 2.

since there is no loss of cholesterol possible during any of the operations.

It is carried out as follows:

About 5 cc. of chloroform are put into the extraction tube

(Fig. 1)[1] and the filter paper disc (Fig. 2)[2] is dropped into the extraction tube; it should lie at point *X*. The constricted portion of the tube, *B*, thus serves a double purpose: it holds the paper disc in place, and permits accurate dilution.

0.25 cc. of oxalated blood is pipetted on the filter paper disc. The blood is immediately absorbed by the filter paper. The extraction tube is then attached to a reflux condenser and immersed in a beaker of hot or boiling water placed over a small electric stove. The water in the beaker should be above the level of the chloroform in the tube.

Extract for 30 minutes. Detach the tube from the condenser, remove filter paper disc from tube, and cool by immersing in cold water for a minute. When cool, add chloroform exactly to the 5 cc. mark.

In a similar extraction tube place 5 cc. of a standard solution containing 0.4 mg. of cholesterol per 5 cc. This standard is made by dissolving 40 mg. of pure dry cholesterol in 500 cc. of pure dry chloroform.

Add 2 cc. of pure dry acetic anhydride and 0.1 cc. of concentrated sulfuric acid to each tube. Insert cork stoppers into the tubes and invert twice to mix well. Place the tubes in a beaker of cold water for half a minute to cool, and leave in a dark place for 10 minutes and read in colorimeter.

The standard is set in the colorimeter at 10 or 15 mm., depending on the intensity of the color of the unknown.

Calculation of Results.

$$\frac{S}{R} \times 160 = \text{mg. per 100 cc. of blood.}$$

S = reading of standard.
R = reading of unknown.

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[1] The entire length of the extraction tube (Fig. 1) is about 4 inches. The upper part *A* is 2 inches long and $\frac{7}{8}$ of an inch in diameter. The constricted part *B* is $\frac{1}{2}$ inch in diameter and $1\frac{1}{4}$ inches long. The capacity of the bulb *C* is such as to hold a trifle less than 5 cc. of fluid, so that when exactly 5 cc. of fluid are placed in the tube, the meniscus of the fluid reaches about to the middle of the constricted portion *B* which is marked by a line.

[2] The filter paper disc (Fig. 2) was prepared from Whatman fat-free extraction thimbles. A number of other filter papers were tried, but these were found to be best for this purpose. The disc has a diameter of $\frac{3}{4}$ inch and a thickness of $\frac{1}{16}$ inch. It has a small opening in the center so that the vapors of chloroform may easily pass.

The following experiments were done to determine the accuracy of this method.

In the first place it was necessary to make sure that the cholesterol is completely extracted by the chloroform from the filter paper. Thus seven discs of filter paper were impregnated with varying amounts of cholesterol, ranging between 0.2 and 1 mg. These discs were dried at 100°C. in an electric oven and extracted with chloroform for 1 hour. Seven controls were set up containing corresponding amounts of cholesterol; *i.e.*, between 0.2 and 1 mg. After developing the color, in the usual way, the extracts were matched in the colorimeter, each against its respective control.

As Table I shows, the cholesterol was completely extracted, since each extract matched its respective control.

TABLE I.

Sample No.	1	2	3	4	5	6	7
Amount of cholesterol on disc, *mg*.	0.2	0.3	0.4	0.5	0.7	0.8	1.0
" " " in control, *mg*.	0.2	0.3	0.4	0.5	0.7	0.8	1.0
Reading of control, *mm*.	15	15	15	15	10	10	10
" " extract, *mm*.	14.9	15	15.2	14.8	9.8	10.3	10

Another experiment was set up with small amounts of blood to which were added varying amounts of cholesterol.

Five filter paper discs were each impregnated with 0.1 cc. of a blood which was found to contain 150 mg. of cholesterol per 100 cc. To each of the discs were added amounts of cholesterol varying between 0.1 and 0.5 mg. The technique used was the one described in the method above. Where the amounts of cholesterol solution were too large to go on the filter paper disc, two discs were used, one placed on top of the other. The standard was made up of 0.4 mg. of cholesterol per 5 cc. of chloroform.

0.1 cc. of the blood, containing 150 mg. per 100 cc. when extracted with 5 cc. of chloroform, is equivalent to 60 mg. of cholesterol per 100 cc.

Each 0.1 mg. of cholesterol added to 5 cc. of choloroform is equivalent to 40 mg. per 100 cc.

Thus, if the extractions were complete, each extract ought to

contain 60 mg. of cholesterol plus 40 mg. of additional cholesterol for each 0.1 mg. added to the disc.

As Table II shows the extractions were complete.

The time it takes to extract completely the cholesterol from the filter paper was found to be 20 minutes, so that by allowing 30 minutes the extraction is certainly complete.

It was further found that it is unnecessary to dry the blood on the filter paper. Extraction with chloroform may begin as

TABLE II.

Sample No.	1	2	3	4	5
Cholesterol used, *mg.*	0.1	0.2	0.3	0.4	0.5
Amount of blood used, *cc.*	0.1	0.1	0.1	0.1	0.1
Cholesterol per 100 cc., *mg.*	95	144	183	212	254
Theoretical values	100	140	180	220	260

soon as the blood is placed on the discs. Drying at high temperatures impairs the test slightly and gives somewhat lower results.

This method was checked against the method of Myers-Wardell[3] on more than a hundred bloods. The results showed, in practically all cases, slightly higher figures by this method. This was to be expected, since the somewhat lower results obtained by the Myers-Wardell method are due to slight losses incurred while removing the dried plaster of Paris to the extraction shell, and while transferring the chloroform extract from the comparatively large flask to the small tube. In the new method here described no such loss is possible since no transfer of material is made.

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THE EFFECT OF ACUTE SCURVY ON THE SUBSEQUENT NUTRITION AND GROWTH OF GUINEA PIGS.*

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The appreciation that certain types of disease have their etiology in dietary deficiencies of various kinds is comparatively recent. Experimental evidence has shown that beri-beri, scurvy, goiter, rickets, and certain types of ophthalmia arise not because of the presence of an infective agent but primarily through the absence of some substance in the diet. The factors thus concerned in preventing or curing this type of affection are potent in such small quantities when compared to the total body mass, that their omission in a dietary may easily be overlooked. The resulting pathological manifestations develop relatively slowly so that a well defined case of deficiency disease usually represents a condition which has become well established.

The more or less permanent lesions in cases of rickets, scurvy, and xerophthalmia, for example, attest the structural alterations following such conditions. That functional disturbances also result from this type of affection is shown by the polyneuritic convulsions in animals suffering a lack of vitamin B, by the breakdown of resistance to infections in vitamin A deficiency, and by the alteration of the normal calcium and phosphate balance in the blood in rickets. These functional disturbances and, in many cases, the structural deformities can be removed by supplying the missing factor in the diet. The loss of appetite, changes in the bone development, and the obscure factors leading to the weakness of the capillary walls, indicate that in scurvy likewise, there is a marked functional maladjustment. Most of the observations

* A preliminary report of this work was made at the annual meeting of the American Society of Biological Chemists, St. Louis, 1923.

have been made on animals or patients in the height of the disease. The question arises, does the cured animal behave normally after having suffered such a tremendous upset?

The symptomatology and pathology of experimental scurvy in guinea pigs has been described in detail by Cohen and Mendel (1918) and by Jackson and Moore (1916). Hemorrhagic gums with loosening of the teeth together with hemorrhages and their sequelæ along the intestinal tract are common enough in the study of scurvy to lead one to suspect that even after the disease is clinically cured, digestion and absorption might be somewhat abnormal.

Certain observations on animals used in another experiment (Smith (1922)) and also the fact that radiographic evidence of scurvy was observed as long as 10 months after the disease had been cured clinically in children,[1] led to the present experiments which were designed to study the effect of scurvy on growth and appetite after apparent cure.

Detailed accounts of accurate observations on metabolism in the postscorbutic stage are few. Lust and Klocman (1912) observed a negative mineral balance (Ca, PO_4, Cl) in the convalescent stage in an infant, while in the period of florid scurvy the balances had been positive. In a study of body weight and body length in infants, Hess (1916) observed that as scurvy developed there occurred a concomitant cessation in weight increase and also a decrease in linear growth. Addition of orange juice or orange peel juice to the diet resulted in prompt resumption of growth at an increased rate as measured by both criteria.

Since the classical work of Holst and Frölich (1907) on experimental scurvy a great number of experiments have been carried out on different phases of this deficiency disease. Undoubtedly all the various diets employed have lacked the antiscorbutic factor, but many of the rations have been so deficient in other respects also that additional complications probably entered into the final results. The diet employed in the present experiments has been demonstrated to suffice for normal growth in the guinea pig when supplemented with a source of vitamin C and for normal growth in the rat without any supplements. We believe that by use of this food we have eliminated complicating nutritive factors in our experiments.

[1] Personal communication of Dr. Elsie J. Dalyell.

EXPERIMENTAL.

Selection of Guinea Pigs.

Each experiment involved the use of two guinea pigs. The individual in which scurvy was induced and with which curative measures were adopted later was designated as the "experimental" animal, while the animal which was at all times provided with an abundant supply of the antiscorbutic factor served as its "control."

Guinea pigs of the same sex, age, weight, and from litters of the same size, were grouped for use in each experiment. The guinea pigs ranged in weight from 150 to 210 gm. at the outset and in each case the "experimental" and "control" animals weighed within 10 gm. of each other. By adopting such a procedure we attempted to eliminate differences in the animals themselves which might complicate our results.

Diets.

The diet fed consisted of the following:

	per cent
Soy bean gruel flour and maysoya[2] (mixed in equal parts)	86
Cod liver oil	5
Dried yeast	3
Calcium lactate	3
Sodium chloride	3

The mixture of soy bean gruel flour and maysoya after being autoclaved for 30 minutes at 15 pounds pressure was spread on glass plates and dried over a radiator for 3 or 4 hours. After the addition and thorough mixing of the other ingredients of the diet, water was added in sufficient quantity to make the mass pack together. This was then dried overnight on glass plates on a radiator. This diet is similar to the soy bean cracker fed by Cohen and Mendel (1918). Cod liver oil as a source of vitamin A was substituted for the raw milk which they employed and paper pulp was omitted because in some preliminary experiments it was observed that the guinea pigs did not consume it. In addition to the soy bean meal 20 gm. of fresh raw cabbage were fed to the

[2] The soy bean gruel flour and the maysoya products were kindly furnished by the Cereo Company, Tappan, New York.

"control" throughout the experiments, while an equivalent quantity of cooked, dried cabbage[3] was fed to the "experimental" animal up to the time that curative measures were introduced. These curative measures consisted of the substitution of 20 gm. of fresh raw cabbage for the cooked, dried cabbage. Fresh water was given *ad libitum* to all guinea pigs.

Procedure.

Preliminary growth rates were obtained on two lots of normal guinea pigs fed on different diets and raised under environmental conditions identical with those of the animals used later. Square metal cages with removable trays or round metal cages similar to those used in rat experiments in this laboratory were employed. All cages were thoroughly cleaned and sterilized at least twice weekly. One lot was fed the soy bean diet *ad libitum* plus 20 gm. of fresh cabbage, while the other lot was fed timothy hay and oats *ad libitum* plus the same quantity of cabbage. Both diets, therefore, furnished an abundant supply of the antiscorbutic dietary factor. Cohen and Mendel (1918) raised guinea pigs which were "maintained in splendid condition on soy bean cracker plus 10 gm. of fresh cabbage daily for more than 90 days." It should be stated that the same care with respect to age, weight, and selection from litters of the same size was exercised with these animals as with the animals used in our scurvy experiments.

We were interested in the comparison of growth curves thus obtained with curves which we constructed from Minot's (1891) figures. These curves are presented in Charts 1 and 2. In an extended study on "Senescence and rejuvenation" Minot (1891) raised a large number of guinea pigs on hay, oats, and carrots, fresh grass being substituted for the carrots in the summer. We raised twelve guinea pigs; three males and three females on each diet.

Having satisfied ourselves that normal growth could be obtained under the experimental conditions with respect to food and environment we proceeded to undertake the experiments as previously outlined in this paper.

For 3 or 4 days after the arrival of the guinea pigs in the

[3] The fresh cabbage was boiled in water for 1 hour and dried in an oven in air at 80–90°C., for 2 days. 0.8 gm. of cabbage thus treated was obtained from 20 gm. of the fresh leaves.

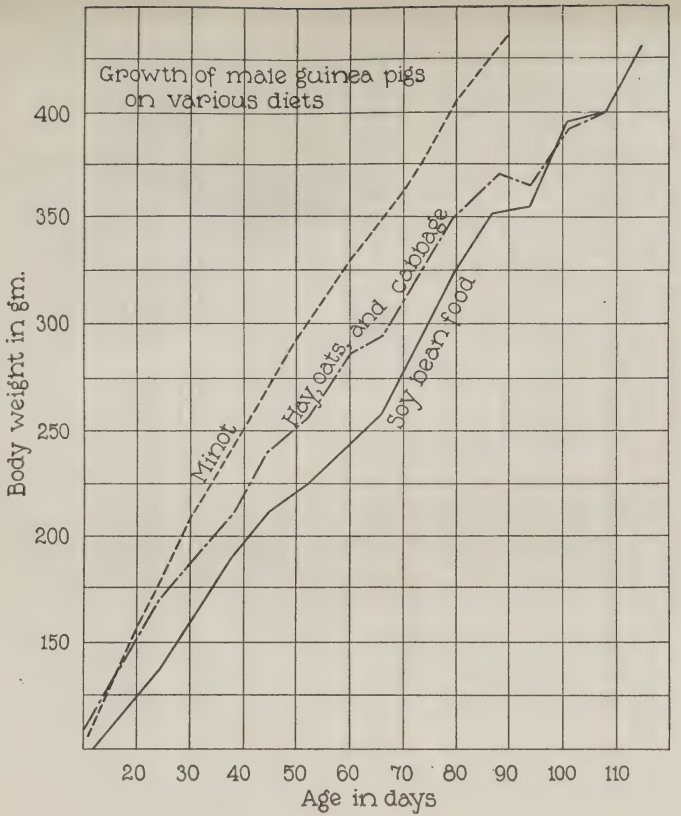

CHART 1.

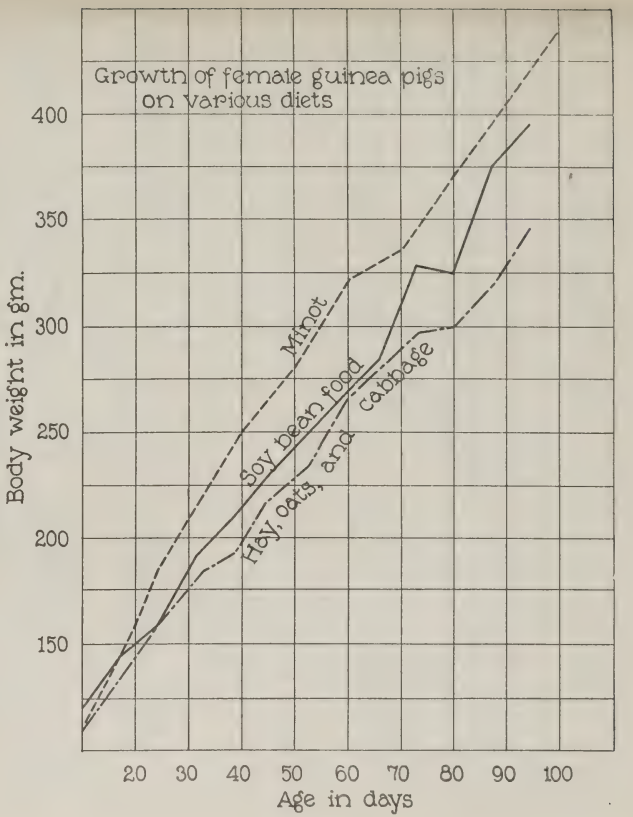

CHART 2.

laboratory all were fed the soy bean meal diet *ad libitum* plus a liberal quantity of fresh raw cabbage. During this period of adjustment to the diet the guinea pigs usually lost weight due in part to the elimination of the bulky intestinal contents from the food fed during the fore period (see Sherman and Smith (1922)). It was after this preliminary interval that the cavies were started on the experiment proper.

Each "experimental" animal was fed the soy bean meal *ad libitum* from weighed portions. The quantity of food consumed daily was determined by weighing the remaining food. The exact amount of food consumed by the "experimental" individual was then weighed out to its control. For example, after having determined the amount of food consumed by the "experimental" cavy during the previous 24 hours, the same amount was fed to its "control" during the next 24 hours. By this method of feeding it is obvious that the effect of inanition *per se* on the growth rate was controlled.

When an "experimental" animal exhibited definite sensitiveness to pressure on the wrists as evinced by squealing and wincing it was pronounced scorbutic. This condition developed in from 10 to 18 days. Tenderness of the muscles of the inner side of the tibia usually developed 2 or 3 days later. We noted a rather prompt swelling of the wrists which hardened within the space of a week's time into exostoses. In two cases the face-ache position described by Chick, Hume, and Skelton (1918) was observed. In another case costochondral enlargements spoken of by Jackson and Moore (1916) were felt, while in still another we were not able to palpate the enlargements, but the guinea pig showed a definite sensitiveness of the rib region.

It was stated above that for 3 days after the "experimental" animal was pronounced scorbutic we continued to feed cooked, dried cabbage. Then the cooked, dried cabbage was replaced by 20 gm. of fresh raw cabbage. In two or three preliminary experiments we found that if we delayed the feeding of fresh raw cabbage for more than 3 days the cavy was unable to eat either the fresh cabbage or the soy bean meal due to the development of sore gums. In these experiments we found it necessary to force feed with the juice of canned tomatoes for 4 or 5 days before the sore gum condition was relieved.

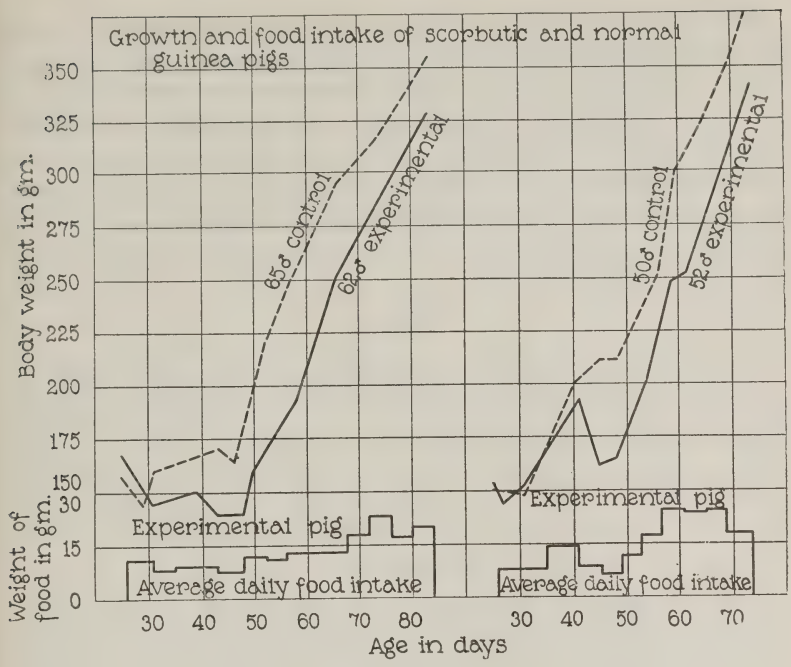

Chart 3.

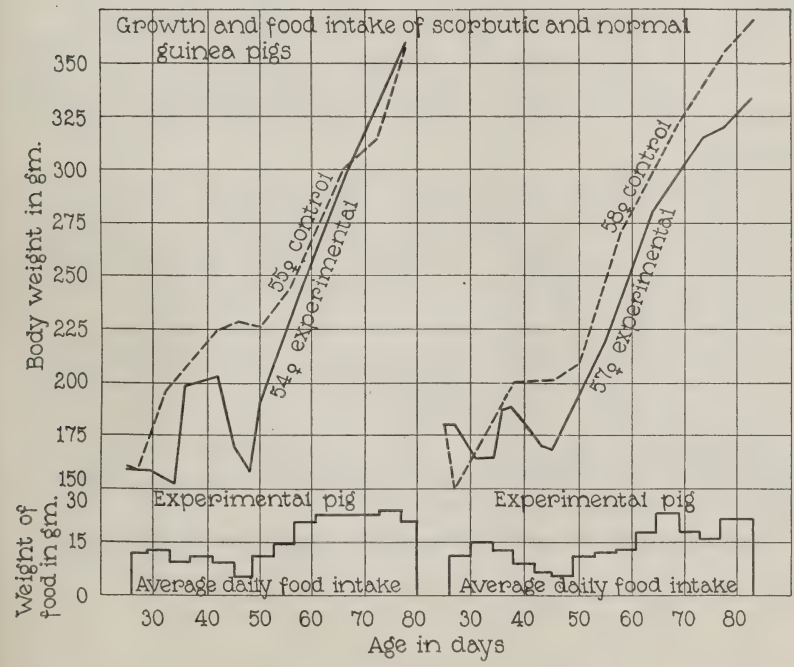

Chart 4.

Five experiments were run 51 to 61 days, while four extended over periods of 103 to 110 days. In about 80 per cent of the cases it was not possible to determine just when the sensitiveness of the wrists and other parts mentioned disappeared. Some of the animals appeared sensitive even at the end of the long experiments. We were strongly suspicious that some of the cavies squealed merely from force of habit when touched, which behavior made it impossible to judge by this criterion just when they became insensitive to pressure.

RESULTS.

The food intake of the "experimental" animals decreased with the onset of scurvy. This fact is clearly shown at the bottom of both Charts 3 and 4 where the food intake throughout the entire experiment is shown. These charts are representative of others that might be given. The food intake of the "experimental" animal from the time that feeding of raw cabbage was begun to the end of the test was compared with that of the "control" for the same period. A distinctly greater food intake for the "experimental" animal was found. The percentage increase of food consumption for the "experimental" animal based on that of its "control" ran all the way from 1.8 to 26.6 per cent in different pairs of guinea pigs. The average increase was between 9 and 16 per cent.

After the "experimental" animals had been given fresh raw cabbage they immediately resumed growth at the same rate as that of their "controls." In two cases their growth rate was greater.

Representative growth curves of two groups of males and two groups of females are shown in Charts 3 and 4. First clinical signs of scurvy in Guinea Pig 62 were elicited at 40 days of age; in Guinea Pig 52, at 42 days; in Guinea Pig 54, at 42 days; and in Guinea Pig 57, at 41 days.

It is interesting to compare the loss in body weight of the "experimental" animals with their "controls." Although both guinea pigs in each group suffered the same diminution of food intake, the "control" animal showed far less loss in weight, which emphasizes the fact that the development of scurvy is itself accompanied by a definite loss of body tissue.

In the light of our previous experience with scurvy in the guinea

pig (Smith (1922)) we feel confident that our "experimental" animals were suffering from this disease. Furthermore, practically all the symptoms described so completely by Cohen and Mendel developed in our animals. We sacrificed only one guinea pig in the height of scurvy as determined by the clinical signs. Autopsy[4] examination revealed subcutaneous and intramuscular hemorrhages throughout the body and a slight increase in fragility of the bones. A faint suggestion of Fraenkel's[5] "white line" at the junction of the epiphysis and diaphysis of the long bones was observed in the x-ray [4] picture taken before death. These observations further served to fortify our conviction that we have produced scurvy in our "experimental" animals. On the other hand, when guinea pigs were photographed by the x-ray 35 and 75 days, respectively, after they had had scurvy, no indication of the disease was manifested. Autopsy on these animals at this time showed no typical lesions.

DISCUSSION.

Bessesen (1923), studying the weights of various organs in guinea pigs suffering from this disease, concluded that the scorbutic condition involves factors influencing body weight not present in fasting. Our experiments confirm his conclusion. During development of scurvy, the "experimental" animals invariably suffered a greater loss in body weight than did the "controls," although their food intakes were quantitatively identical.

Studies of vitamin B deficiency in dogs by Cowgill (1921) have shown that loss in body weight associated with this condition is concomitant with a diminished food intake; whether or not this loss was due simply to failure to eat was not determined. Our investigations in vitamin C deficiency in guinea pigs lead us to believe that there are factors resulting in loss of body weight, which operate in addition to fasting, and which depend on scurvy itself. In other words, inanition alone does not cause as severe a loss as when accompanied by lack of vitamin C.

[4] We are indebted to Dr. G. F. Powers, Department of Pediatrics, Yale Medical School, who performed the autopsies, and to Dr. C. R. Scott and Mr. E. F. Furbush, X-ray Department, New Haven Hospital, who took the x-ray pictures.

[5] Quoted by Gerstenberger (1918).

The curative measures adopted in our work appeared promptly to remove pathological evidences of *acute* scurvy previously produced in the "experimental" animals. We know, however, from a former rather extensive experience (Smith (1922)) that in *chronic* scurvy numerous lesions result and nutritive disturbances are observed. Changes in bone structure would undoubtedly be seen in x-ray pictures of such cases. Our results impress us with the truth of Minot's (1891) statement that "to permanently dwarf a guinea pig requires an astonishingly prolonged interference." In this connection the immediate response to curative measures noted in our "experimental" animals deserves comment.

It was observed above that after scurvy the "experimental" animals ate distinctly more of the soy bean ration than their "controls." It is conceivable that under the influence of added vitamin C, total metabolism is increased, with the result that, in order to make the "normal" gain observed after scurvy, additional food must be ingested. Again it appears that at least the digestive and absorptive mechanism of the scorbutic guinea pigs suffered a definite damage in the course of the experiment since in order to make gains comparable with the normal animals an increased food intake was necessary.

SUMMARY.

Experiments were conducted to determine the effect of severe, acute scurvy on the subsequent growth and food intake of guinea pigs. It was convincingly demonstrated by the carefully controlled methods employed that the loss of body weight observed in the present series of scorbutic guinea pigs at the height of scurvy cannot be accounted for by the inanition factor alone. Growth in the postscorbutic stage is either parallel to or greater than that of the normal guinea pig and is accompanied by a definite increase of food intake.

Acknowledgment is due Miss Elizabeth B. Carey for valuable assistance rendered in the course of the experiments.

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INVERTASE IN HONEY.*

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The invertase which has been the subject of the extensive researches of the past 50 years has almost invariably been obtained from yeast. Sucrose-splitting enzymes are, however, of widespread occurrence. Aside from yeast, several other species of fungus contain them (1, 2), and sucroclastic enzymes have also been found in the roots and fruits of higher plants (3–5), in certain forms of bacteria (6), and in the tissues and digestive secretions of some insects (7), birds (8), and mammals (9).

The question has naturally arisen from time to time, as to whether the invertases from such varied sources are identical, or whether each one is especially adapted to the peculiar conditions under which it is called upon to exert its influence.

In the case of other enzymes besides invertase, the same problem has, of course, also presented itself, and a number of comparative studies have been reported in the literature. The most far reaching of these comparisons is that of Sherman and his students (10–16), who investigated the action of amylases derived from the pancreas, the saliva, malt, and takadiastase. The properties of these amylases do not exactly coincide. Nevertheless the investigators found enough parallelism in action, to lead them to the conclusion that, while not identical, all these amylases are similarly constituted, and that the mode of attack upon the substrate, starch, is the same in all cases.

Comparative studies have also been made, by Northrop (17) and others (18–20), upon the proteases obtained from the various digestive secretions of mammals, as well as from certain plants.

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These investigations have led to conclusions similar to those cited for the amylases.

The starches and the proteins are complex substances, and contain several kinds of hydrolyzable linkages within the molecule. One of the ways in which the enzymes from various sources, but specific to the same substrate, differ from one another, is that they do not affect corresponding linkages in the substrate in the same way. Thus, pepsin splits proteins only as far as the peptone stage; trypsin, on the other hand, hydrolyzes proteins all the way to amino acids. In the case of the amylases from several plant and animal sources, the ratio of the amylolytic, or starch-splitting power, to the saccharogenic, or sugar-forming power, varies between 9 to 1, and 2 to 7.

These complexities leave the question open as to whether enzymes from different sources, specific to the same linkage within the same compound, bring about the hydrolysis of that linkage in exactly the same manner. For, since the hydrolysis of the several kinds of linkages within the starch or protein molecule all proceed simultaneously, it would be difficult to trace experimentally the progress of the reaction with respect to any one linkage singled out for study.

In order, therefore, to determine whether enzymes from different sources, which are specific to the very same linkage in the same compound, act in exactly the same manner or not, it is desirable to compare the action of different preparations of enzymes acting upon a substrate in which there is only one linkage subject to the hydrolytic action. There are numerous cases of this kind, such as the hydrolysis of maltose by maltase, or of lactose by lactase. Several reports concerning studies of maltases and lactases (21) derived from different plant and animal sources are in the literature. In most instances, however, the enzyme preparations did not suffice for accurate and exhaustive investigations, and the errors involved in following the reaction were very large. Moreover, the effect of hydrogen ion concentration, which is of prime importance in enzyme work, was usually neglected. As a rule, too, the experiments were not planned with the object directly in view of comparing the actions of the enzymes from different sources, and hence hardly any strictly comparable data are available.

The hydrolysis of simple esters by the lipases has been studied, however, with a view of comparing the action of enzymes from different sources. Thus, very recently, Willstätter and Memmen (22) have worked with pancreas and stomach lipases, and have come to the conclusion that certain apparent differences in their action are due to removable accompanying substances. The comparison was confined to a study of the effect of hydrogen ion concentration upon the rate of hydrolysis, and the experimenters were hindered by the difficulty that the stomach lipases which they used could only be obtained in preparations which were very weak, compared with those obtainable from the pancreas. There are, moreover, several difficulties inherent in the work with the esterases. The esters are only partially soluble in water, and this puts limitations on the study of the effect of the enzymes upon solutions containing various substrate concentrations. Furthermore, the esters are partially hydrolyzed by the buffer solutions used to control the hydrogen ion concentration, and, besides, the enzymes need activating salts which are not the same in all cases. These factors interfere with the obtaining of exactly comparable conditions in the study of lipases from various sources.

Invertases, on the other hand, offer marked advantages for comparative studies of this kind. Their substrate, cane-sugar, also contains only one linkage which these enzymes attack. But cane-sugar, in contrast to the substrates mentioned above, is easily soluble in water, and readily obtainable in a high degree of purity. Moreover, the course of the inversion of sucrose can be very conveniently and accurately followed by means of the polariscope. A further distinct advantage that a study of invertases for comparative purposes presents, is that one invertase, that derived from bottom yeast, has been the subject of a large number of investigations. Very much of this work, especially in recent years, has been done under well standardized conditions, and a large mass of detailed information concerning yeast invertase is therefore available, with which the results of investigations upon an invertase from another source may be compared.

But despite these advantages offered by a comparative study of invertases, the object of most investigations upon other sources than yeast has been merely to ascertain the presence of an invertase in the organism studied. This is in large measure due to

the difficulties which have heretofore been encountered in obtaining invertase preparations suitable for extended work, from any source except yeast. Thus, while Falk and McGuire (4) did considerable work upon the invertase present in bananas, and von Euler and Svanberg (9) upon that present in the intestines of mammals, the natures of the invertase preparations used unfortunately were not such as to permit of as exact measurements as have been possible with yeast invertase.

For the present research an invertase was sought, which could be obtained, just like yeast invertase, in clear, stable solutions. A search of the literature pointed to honey as the most likely source. Honey had been variously reported to contain a sucroclastic enzyme, and although nothing had been done to ascertain its properties and mode of action, one investigator had indicated that an active precipitate could be obtained when honey was treated with alcohol (7).

A method has now been developed for obtaining clear, aqueous solutions containing honey invertase, which are comparable to yeast invertase preparations both as regards clearness and stability. The crude preparations obtained by alcohol precipitation are amenable to purification by dialysis, and by other methods which are used in yeast invertase work. The honey invertase preparations procurable in this way are, moreover, comparable in strength to those ordinarily employed in the study of yeast invertase.

These favorable properties cause honey invertase to lend itself readily to the duplication of previous experiments conducted with the yeast product. We have here a very fortunate case of an invertase obtained from an entirely different source, which can be studied with exactly the same methods of procedure as are applied in the case of yeast invertase. In the present study, there have been compared some of the characteristics of these two enzymes, the one derived from the fungus, yeast, and the other from an animal source, the digestive secretions of an insect, the bee.

EXPERIMENTAL PART.

Preparation of Honey Invertase.

It was found that the physical properties of the honey invertase preparations, especially the clearness, depended both on the

methods of precipitation and filtration, and on the kind of honey used as the starting material. Attempts to obtain a sucroclastic enzyme from canned "Orange Blossom Honey" were entirely unsuccessful. From combs of "White Clover Honey" active preparations were isolated, but these were too cloudy to permit of solutions containing them being read in the polariscope. Continued efforts to clarify these preparations with charcoal or kaolin were in vain. Where clear solutions did result from this treatment, they were either inactive, or showed only slight activity. It was finally found, however, that clear, active preparations could be obtained from a product called "Buckwheat Honey." This material, which is very dark in color, is available in the open market in small combs weighing about 400 gm. each, designed for table use.

These combs were thoroughly mashed, and treated with about 150 cc. each of distilled water. That part of the beeswax which rose to the surface was skimmed off; the remainder was separated by suction filtration. To the filtrate were added 7 to 10 volumes of 90 per cent ethyl alcohol. The precipitated gums settled fairly rapidly; most complete settling was found to occur when the full quantity of alcohol was added at one time. The supernatant liquid was then siphoned off, and what was left was drained through fluted filters. For this operation suction filtration is unsuitable, for when it is used a cloudiness results, which cannot be easily removed by subsequent treatment. The gummy residue was in the main insoluble in water, but when stirred with the latter and filtered, yielded clear solutions containing active invertase. If fairly active preparations are desired, not more than 10 cc. of water to each comb originally taken should be used for this washing.

The first preparation procured in this way was employed for preliminary experiments, without purification. All subsequent preparations were dialyzed in collodion bags against running water, for from 48 to 72 hours, in order to remove any contaminating salts or invert sugar. All solutions were treated with small amounts of toluene to prevent bacterial growth.

When the above procedure is carried out, an average yield of about 10 cc. of invertase solution is obtained from each comb. This amount of enzyme solution, when present in 100 cc. of a 10

per cent sucrose solution at 25°C., at the optimum hydrogen ion concentration, brings about the inversion of 5 gm. of sucrose in about $2\frac{1}{2}$ hours.

Comparison of Certain Properties of the Invertases in Honey and Yeast.

Since varying results have always been obtained in attempts to determine the chemical constitution of yeast invertase by direct analysis, and no definite correlation has been shown between the composition and activity of different yeast invertase preparations, it was thought best to avoid this method for the time being in the study of honey invertase. Instead, the experiments were devoted entirely to determining the characteristics of inversions in the presence of honey invertase, and, for purposes of comparison, those characteristics of honey invertase were studied, of which the analogues in the case of yeast invertase are best known. Such characteristics are:

1. The course of hydrolysis of cane-sugar with respect to time at various hydrogen ion concentrations.
2. The range of hydrogen ion concentration over which the enzyme shows the greatest activity.
3. The stability of the enzyme at various hydrogen ion concentrations.
4. The effect of various initial sucrose concentrations upon the course of hydrolysis.

Method.

Before dealing exhaustively with the results of the experiments with honey invertase, undertaken to determine the characteristics of its inversions enumerated above, it is necessary to describe briefly the procedure followed, which is an application of the standard methods employed in following enzymatic sucrose inversions.

Definite quantities of honey invertase preparations were added to sucrose solutions of known concentration at a temperature of 25°C. The hydrogen ion concentrations of these solutions were controlled by means of sodium citrate buffers, and determined electrometrically. At measured time intervals during the course of the hydrolyses, samples were withdrawn from each of the reaction mixtures, and treated with sodium carbonate to interrupt

the inversion. The samples were then polarized to ascertain what part of the hydrolysis had taken place.

A few preliminary experiments showed that the activity range of honey invertase centers about a hydrogen ion concentration of about 10^{-6}, and the investigations were therefore conducted at hydrogen ion concentrations varying to both sides of this point.

Several invertase preparations were used in these studies, each obtained from honey purchased at different times over a period of 3 months. The experiments performed with any one preparation were partially repeated with one or more of the others. By this means the possibility was precluded that the phenomena observed were peculiar to any one preparation, and not generally characteristic of honey invertase. In the data, the following designations are used for these preparations: "HA" (not dialyzed); "HB" (dialyzed 48 hours); "HC" (dialyzed 72 hours); "HD" (dialyzed 60 hours).

Course of Hydrolysis of Sucrose in the Presence of Honey Invertase.

The results recorded in Tables I to IV, and plotted in Figs. 1 to 5, represent hydrolyses of 10 per cent sucrose solutions in the presence of honey invertase. The 10 per cent concentration was chosen because the most extensive work in this laboratory with yeast invertase, was done with sugar solutions of this concentration. The hydrolyses were run at hydrogen ion concentrations ranging from $10^{-3.95}$ to $10^{-7.60}$. Four different invertase preparations were used, and the rate of hydrolysis was varied considerably by the adjustment of the amount of invertase taken for the experiments of each series. The preparation, "A", "B", "C", or "D", which was used for each series of hydrolyses, is recorded in the data, and on the graphs.

Description of the Course of Hydrolysis.—Despite all the variations in conditions noted in the preceding paragraph, all these hydrolyses manifest certain outstanding features, which they have in common. Thus, immediately after the incipiency of the inversion, there is a marked increase in the reaction velocity, which lasts until a maximum is attained—generally between 10 to 15 per cent hydrolysis—and then a gradual fall in velocity sets in. Each curve in Figs. 1 to 5, therfore, shows first a portion (marked "X" in Fig. 5) convex to the abscissa, then a point of inflection ("Y"), and, finally, a concave portion (marked "Z").

TABLE I.

Course of Hydrolysis and Hydrogen Ion Concentration.

5 cc. of honey invertase preparation, "HC", in 225 cc. of solution.
Sucrose concentration: 10 gm. per 100 cc.
Temperature: 25°C.
Samples: 25 cc.
Na_2CO_3 (0.1M) : 5 cc.
Polariscope tube: 400 mm.

pH	Time.	Observed rotation.	Change in rotation.	Hydrolyzed.	$k \times 10^3$
	min.			*per cent*	
3.95	0	25.90			
	67½	25.83	0.07	0.21	13.3
	135	25.67	0.23	0.69	22.2
	385	25.16	0.74	2.21	25.2
	1,335	22.44	3.46	10.30	36.0
	2,914	18.95	6.95	20.70	34.5
	5,670	14.95	10.95	32.60	30.2
	8,586	12.40	13.50	40.22	27.2
	Final.	−7.66	33.56		
4.25	0	25.90			
	67½	25.67	0.23	0.69	66.2
	135	25.41	0.49	1.46	70.9
	270	24.74	1.16	3.46	75.3
	1,485	18.25	7.65	22.79	87.9
	2,840	13.20	12.70	37.83	86.1
	4,370	9.26	16.64	49.60	66.1
	7,600	3.70	22.20	64.65	59.4
	Final.	−7.67	33.57		
4.69	0	25.89			
	67½	25.89	0.29	0.86	54.8
	135	25.15	0.74	2.21	71.9
	270	24.10	1.79	5.33	89.3
	1,060	17.77	8.12	24.20	114
	2,500	10.57	15.32	45.64	106
	4,045	5.90	19.99	59.70	97.6
	5,850	2.49	23.40	69.71	88.8
	Final.	−7.66	33.55		
5.23	0	25.89			
	67½	25.46	0.43	1.28	83.0
	135	24.85	1.04	3.10	102
	270	23.36	2.53	7.54	109
	1,060	15.63	10.26	30.56	150
	2,500	8.22	17.67	52.65	130
	4,045	3.75	22.14	65.97	120
	5,850	0.65	25.24	75.16	104
	Final.	−7.68	33.57		

TABLE I—*Continued.*

pH	Time.	Observed rotation.	Change in rotation.	Hydrolyzed.	$k \times 10^6$
	min.			*per cent*	
5.67	0	25.91			
	67½	25.34	0.57	1.70	111
	135	24.57	1.34	3.99	131
	270	22.75	3.16	9.42	159
	540	19.26	6.65	19.82	178
	1,365	12.41	13.50	40.22	164
	3,495	4.32	21.59	64.33	128
	5,635	0.49	25.42	75.71	109
	Final.	−7.67	33.58		
6.25	0	25.91			
	67½	25.30	0.61	1.82	119
	135	24.55	1.36	4.05	133
	270	22.56	3.35	9.98	169
	515	19.52	6.38	19.01	178
	1,535	11.53	14.38	42.84	158
	2,825	6.32	19.59	58.37	135
	4,460	2.51	23.40	69.71	119
	7,620	−1.45	27.36	81.56	99.3
	Final.	−7.63	33.54		
6.65	0	25.90			
	67½	25.36	0.54	1.61	104
	135	24.76	1.14	3.37	110
	385	21.71	4.19	12.48	150
	1,265	14.25	11.65	34.72	144
	1,895	10.95	14.95	44.56	131
	3,365	6.10	19.80	59.02	114
	5,640	1.83	24.07	71.71	80.2
	Final.	−7.66	33.56		
6.99	0	25.92			
	67½	25.46	0.46	1.37	88.9
	135	24.89	1.03	3.07	101
	270	23.58	2.34	6.97	116
	1,385	15.20	10.72	31.94	121
	2,765	9.89	16.03	47.75	102
	4,340	5.74	20.18	60.12	92.0
	7,492	0.96	24.96	74.30	78.8
	Final.	−7.66	33.58		

TABLE I—*Concluded.*

pH	Time.	Observed rotation.	Change in rotation.	Hydrolyzed.	$k \times 10^6$
	min.			*per cent*	
7.60	0	25.91			
	$67\frac{1}{2}$	25.74	0.17	0.51	32.6
	135	25.57	0.34	1.01	32.6
	270	25.15	0.76	2.26	36.6
	1,380	21.05	4.86	14.47	49.1
	2,760	15.89	10.02	29.85	55.8
	4,325	11.90	14.01	41.74	54.2
	7,492	5.48	20.43	60.85	41.2
	Final.	−7.65	33.56		

TABLE II.

Course of Hydrolysis and Hydrogen Ion Concentration.

8 cc. of honey invertase preparation, "HD", in 225 cc. of solution.
Sucrose concentration: 10 gm. per 100 cc.
Temperature: 25°C.
Samples: 25 cc.
Na_2CO_3 (0.1M) : 5 cc.
Polariscope tube : 400 mm.

pH	Time.	Observed rotation.	Change in rotation.	Hydrolyzed.	$k \times 10^6$
	min.			*per cent*	
4.15	0	25.91			
	40	25.61	0.30	0.89	97.5
	80	25.20	0.71	2.12	
	120	24.74	1.17	3.49	
	180	24.06	1.85	5.51	
	300	22.43	3.48	10.38	
	525	19.44	6.47	19.28	177
	1,435	11.07	14.84	44.23	176
	Final.	−7.67	33.58		
4.60	0	25.92			
	40	25.41	0.51	1.25	168
	80	24.76	1.16	3.46	
	120	23.98	1.94	5.78	
	180	22.75	3.17	9.45	
	300	20.17	5.75	17.14	272
	525	15.99	9.93	29.59	
	1,435	6.46	19.46	57.98	262
	Final.	−7.67	33.59		
5.25	0	25.91			
	40	24.97	0.94	2.81	308
	80	23.86	2.05	6.11	
	120	22.55	3.36	10.01	

TABLE II—*Concluded.*

pH	Time.	Observed rotation.	Change in rotation.	Hydrolyzed.	$k \times 10^6$
	min.			*per cent*	
5.25	300	17.07	8.84	26.34	432
	525	12.31	13.60	40.52	
	1,480	2.62	23.29	69.40	347
	Final.	−7.65	33.56		
5.75	0	25.89			
	40	24.87	1.02	3.04	335
	80	23.44	2.45	7.30	
	120	21.89	4.00	11.92	
	180	19.62	6.27	18.68	
	300	15.88	10.01	29.83	513
	525	11.11	14.78	44.05	
	1,435	2.05	23.84	71.03	375
	Final.	−7.65	33.54		
6.15	0	25.91			
	40	24.86	1.05	3.13	345
	80	23.44	2.47	7.36	
	120	21.92	3.99	11.90	
	180	19.70	6.21	18.50	
	300	16.06	9.85	29.35	503
	525	11.34	14.57	43.42	
	1,435	2.52	23.39	69.69	361
	Final.	−7.64	33.55		
6.42	0	25.90			
	40	24.82	1.08	3.22	355
	80	23.46	2.44	7.27	
	120	22.03	3.87	11.53	
	300	16.48	9.42	28.07	477
	525	11.95	13.95	41.57	
	1,480	2.85	23.05	68.69	341
	Final.	−7.65	33.55		
6.78	0	25.91			
	40	25.03	0.88	2.62	288
	80	23.92	1.99	5.93	
	120	22.70	3.21	9.57	
	180	20.92	4.99	14.87	
	300	17.79	8.12	24.20	401
	525	13.47	12.44	37.07	
	1,435	4.56	21.35	63.62	306
	Final.	−7.66	33.57		

TABLE III.

Course of Hydrolysis and Hydrogen Ion Concentration.

25 cc. of honey invertase preparation, "HB", in 250 cc. of solution.
Sucrose concentration: 10 gm. per 100 cc.
Temperature: 25°C.
Samples: 25 cc.
Na_2CO_3 (0.1M) : 5 cc.
Polariscope tube : 400 mm.

pH	Time.	Observed rotation.	Change in rotation.	Hydrolyzed.	$k \times 10^5$
	min.			*per cent*	
5.70	0	25.91			
	30	22.00	3.91	11.65	276
	60	17.25	8.66	25.81	
	90	13.69	12.22	36.42	
	140	9.60	16.31	48.61	353
	190	6.72	19.19	57.19	
	280	3.17	22.74	67.76	
	400	0.22	25.69	76.54	186
	600	−2.64	28.55	85.09	
	Final.	−7.64	33.55		
5.18	0	25.91			
	15	24.36	1.55	4.62	
	30	22.71	3.20	9.54	144
	60	19.85	6.06	18.06	
	90	15.39	10.52	31.34	
	140	11.06	14.85	44.37	174
	235	5.82	20.09	59.87	
	400	0.90	25.01	74.77	149
	600	−2.32	28.23	84.12	
	Final.	−7.66	33.57		
6.52	0	25.91			
	15	24.30	1.61	4.80	
	30	22.32	3.59	10.79	164
	60	19.52	6.39	19.04	
	90	15.50	10.41	31.02	
	140	11.65	14.26	42.49	179
	235	6.94	18.97	56.53	
	400	2.20	23.71	70.65	133
	600	−1.00	26.91	80.21	
	Final.	−7.66	33.57		

TABLE IV.

Course of Hydrolysis and Hydrogen Ion Concentration.

10 cc. of honey invertase preparation, "HA", 10 gm. of sucrose, 10 cc. of buffer solution, and 80 cc. of H_2O.

Temperature: 25°C.

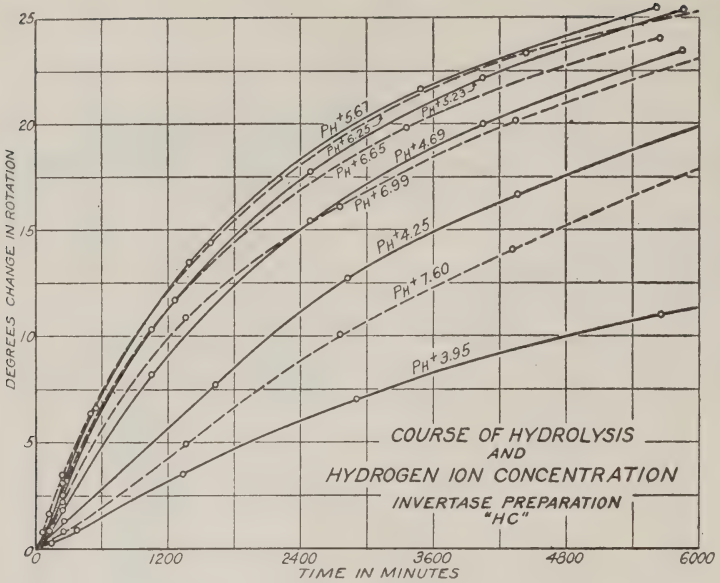
Samples : 25 cc.

Na_2CO_3 (0.1M) : 5 cc.

Polariscope tube: 200 mm.

Initial reading (extrapolated): 11.60°. Final: −3.40°.

pH	Time.	Observed rotation.	Change in rotation.	Hydrolyzed.	$k \times 10^5$
	min.			*per cent*	
4.67	60	9.44	2.16	14.40	113
	135	7.25	4.35	29.00	110
	255	4.77	6.83	45.33	103
5.21	60	8.68	2.92	19.47	157
	135	6.09	5.51	36.74	147
	255	3.52	8.08	53.87	132
5.82	60	8.09	3.51	23.40	193
	135	5.37	6.23	41.53	173
	255	2.90	8.70	58.00	148
6.38	60	8.27	3.33	22.20	182
	135	5.68	5.92	39.47	162
	255	3.27	8.33	55.52	138
6.70	60	8.40	3.20	21.33	174
	135	5.90	5.70	38.01	154
	255	3.52	8.08	53.86	132
7.32	60	9.26	2.34	15.60	123
	135	7.28	4.32	28.80	109
	255	4.99	6.61	45.09	102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TIME IN MINUTES
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PH+6.25
PH+6.65
PH+5.23
PH+4.69
PH+6.99
PH+4.25
PH+7.60
PH+3.95
COURSE OF HYDROLYSIS AND HYDROGEN ION CONCENTRATION
INVERTASE PREPARATION "HC"

Fig. 1.

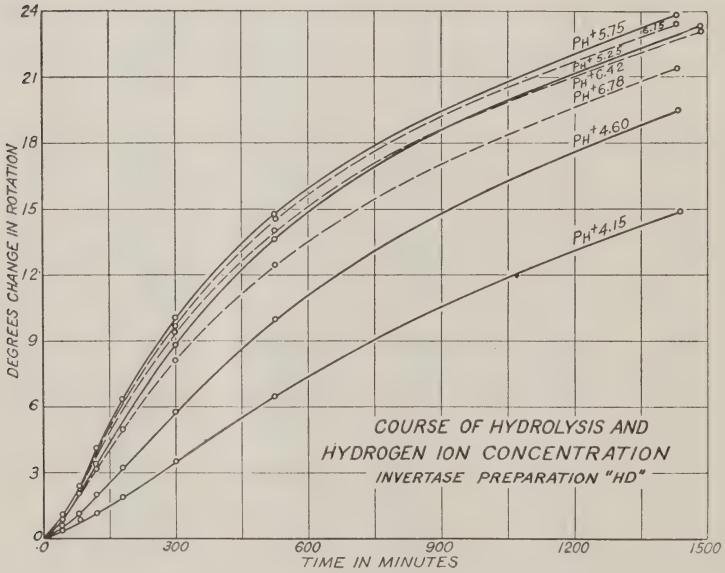
DEGREES CHANGE IN ROTATION
TIME IN MINUTES
PH+5.75
PH+6.15
PH+5.25
PH+6.42
PH+6.78
PH+4.60
PH+4.15
COURSE OF HYDROLYSIS AND
HYDROGEN ION CONCENTRATION
INVERTASE PREPARATION "HD"

Fig. 2.

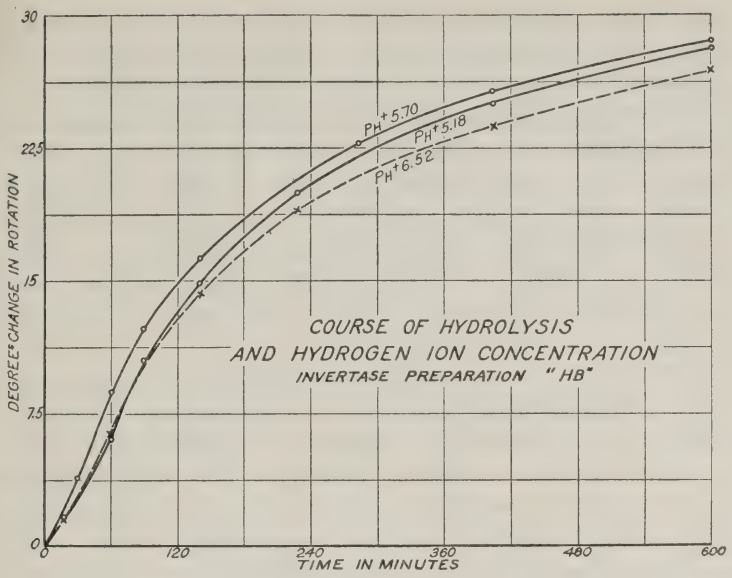
30
22.5
15
7.5
0
DEGREE* CHANGE IN ROTATION
0
120
240
360
480
600
TIME IN MINUTES
PH 5.70
PH 5.18
PH 6.52
COURSE OF HYDROLYSIS
AND HYDROGEN ION CONCENTRATION
INVERTASE PREPARATION "HB"

FIG. 3.

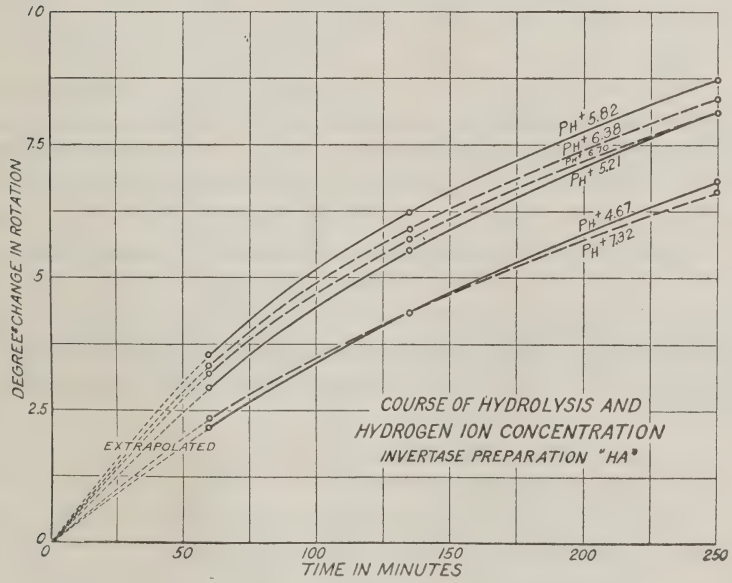
10
7.5
5
2.5
0
DEGREE* CHANGE IN ROTATION
0
50
100
150
200
250
TIME IN MINUTES
PH 5.82
PH 6.38
PH 5.52
PH 5.21
PH 4.67
PH 7.32
EXTRAPOLATED
COURSE OF HYDROLYSIS AND
HYDROGEN ION CONCENTRATION
INVERTASE PREPARATION "HA"

FIG. 4.

This course of hydrolysis, which inversions with honey invertase follow, is very different from that followed by inversions in the presence of yeast invertase. Yeast invertase inversions of 10 per cent sucrose solutions proceed at a practically constant rate at the beginning of the reaction, instead of showing the marked increase in rate found at the beginning of honey invertase hydrolyses. After the first portion of the hydrolysis is complete, the rate of inversion with yeast invertase falls off, in a manner similar to the behavior of honey invertase inversions, but, as will be shown below, the rate of the decline in the velocity of hydrolysis differs in the case of the two invertases.

The Monomolecular Equation Is not Applicable.—Many workers in the invertase field apply the monomolecular equation in describing and comparing hydrolyses of sucrose with invertase. The monomolecular equation, though it fits acid hydrolyses of sucrose, is not so satisfactory for invertase hydrolyses, and its use in comparing such hydrolyses is necessarily crude. Nevertheless, since so much of the work recorded in the literature has been done on this basis, it was thought best to apply the monomolecular equation to honey invertase as well, so as to make the data for the latter available for comparison with the enzyme studies of the past.

In Column 6 of Tables I to IV are tabulated the monomolecular "constants" obtained by an application of the equation to the results given by honey invertase hydrolyses. The monomolecular constants rise rapidly at first, then diminished somewhat more gradually; hence, the inversions are not simple first order reactions.

The monomolecular constants calculated for yeast invertase hydrolyses have been shown almost invariably to rise steadily to the very end of the reaction, instead of showing the rise and fall characteristic of honey invertase inversions. This difference in behavior can be seen from the fourth column in the following examples:

*Yeast Invertase.**

10 per cent sucrose solution. pH 4.65. Polariscope tube : 200 mm.

Time.	Observed rotation.	Inversion.	$k \times 10^5$	$n \times 10^5$
min.		*per cent*		
0	12.03			
30	10.33	10.91	167	259
60	8.68	21.50	175	261
122	5.61	41.21	186	260
200	2.45	61.49	207	260
305	−0.38	79.65	227	260

Honey Invertase.

10 per cent sucrose solution. pH 5.70. Polariscope tube : 400 mm.

Time.	Observed rotation.	Inversion.	$k \times 10^5$	$n \times 10^5$
min.		*per cent*		
0	25.91			
30	22.00	11.66	179	276
60	17.25	25.81	216	317
140	9.60	48.61	295	353
190	6.72	57.19	194	247
400	0.22	76.54	157	186

*See Nelson and Vosburgh (23).

The Nelson-Hitchcock Equation Is not Applicable.—In the case of the invertase from bottom yeast, Nelson and Hitchcock (24) were able to evolve an empirical equation,

$$t = \frac{1}{n}\left(\log \frac{100}{100\text{-}p} + 0.002642p - 0.000008860p^2 - 0.0000001034p^3\right)$$

which accurately represents the course of inversion of 10 per cent sucrose solutions in the presence of this enzyme, over a wide range of hydrogen ion concentrations. It is quite evident from what was said above, that this equation does not hold for the very different course of reaction followed by inversions with honey invertase. This is confirmed by the variation of the constants, "n," obtained by an application of the Nelson-Hitchcock equation to a hydrolysis with honey invertase, recorded in the fifth column of the table given in the preceding paragraph.

Course of Hydrolysis and Hydrogen Ion Concentration.

The manner in which inversions in the presence of honey invertase progress, is influenced considerably by the hydrogen ion concentration of the solution. This can be shown best by means of the curves in Fig. 1, representing inversions conducted at hydrogen ion concentrations of $10^{-5.67}$ and $10^{-6.25}$, respectively. These curves show that the hydrolysis at a hydrogen ion concentration of $10^{-6.25}$, though starting off more rapidly than does that at $10^{-5.67}$, slows up much more than does the latter, once

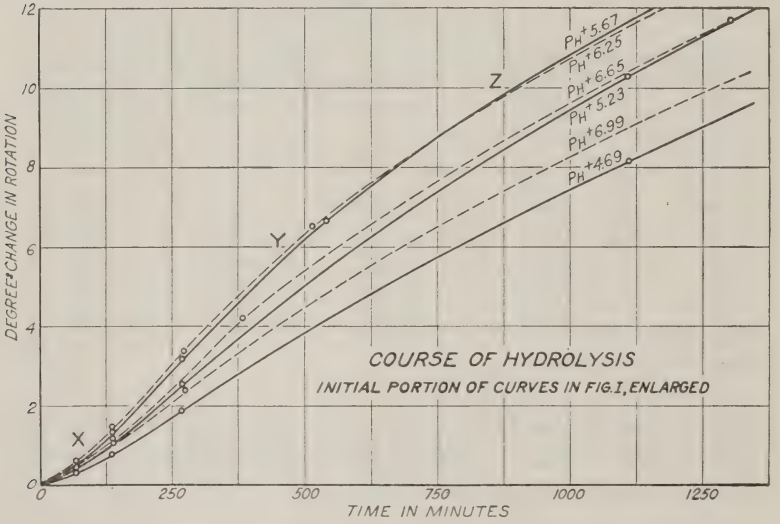

Fig. 5.

the initial stage has been passed; in fact, the curves representing these hydrolyses actually cross each other. An inspection of the rest of the curves in Figs. 1 to 4, shows that the inversions in which the solutions are more alkaline than a hydrogen ion concentration of 10^{-6} decline more rapidly after the first stages of the hydrolysis are over, than do corresponding inversions at higher acidities. To bring this out more clearly in the graphic representation, all the curves in Figs. 1 to 4, which represent inversions at hydrogen ion concentrations alkaline to 10^{-6} are drawn with broken lines, whereas the inversions at higher acidities are represented by solid line curves.

This non-uniformity at different hydrogen ion concentrations, of the course followed by inversions of 10 per cent sucrose solutions in the presence of honey invertase, is in marked contrast with the behavior of hydrolyses where yeast invertase is present. The Nelson-Hitchcock equation, cited above, has been found to hold for hydrolyses of 10 per cent sucrose solutions with the invertase derived from bottom yeast, over a wide range of hydrogen ion concentrations. All these yeast invertase hydrolyses, therefore, follow a uniform course. In other words, the curves representing them can be superimposed, as Nelson and Hitchcock showed, by simply adjusting the time scale. On the other hand, the curves representing honey invertase hydrolyses at various hydrogen ion concentrations are not superimposable.

Activity and Hydrogen Ion Concentration.

The next point we will consider is the range on the hydrogen ion scale, over which the hydrolyses in the presence of honey invertase show the highest rate of inversion. In the case of yeast invertase, the relative magnitudes of the velocity constants given by the Nelson-Hitchcock equation present a convenient method for comparing the rates of inversion at different hydrogen ion concentrations. However, since honey invertase hydrolyses do not follow a uniform course at all hydrogen ion concentrations, it was impossible to derive an equation for honey invertase, similar to the Nelson-Hitchcock equation. It was, therefore, necessary to employ another method, used by Michaelis and Davidsohn (25), Nelson and Vosburgh (23), and others; namely, a comparison of the amount of hydrolysis which has taken place in a given time in each of the inversions.

The experimental data recorded in Tables I to IV, and graphically represented in Figs. 1 to 4, can be used for this purpose. The times (in minutes) required for the hydrolysis of the first 10 per cent of the sugar present, were read off from Figs. 1 and 2. These numbers were then converted into values representing the relative velocities, by setting the smallest number equal to 100, and assigning reciprocally proportionate values to the others.

The Optimum Hydrogen Ion Range Differs for Yeast and Honey Invertases.—The values resulting from the above procedure are tabulated in Tables V and VI, and plotted against hydrogen

TABLE V.

Activity and Hydrogen Ion Concentration.

pH	Time for 10 per cent hydrolysis.	Proportional Nos.	Time for 35 to 55 per cent hydrolysis.	Proportional Nos.
Honey invertase preparation, "HC," data from curves in Fig. 1.				
	min.		*min.*	
4.25	695	41	2,628	54
4.69	467	60	1,821	76
5.23	348	81	1,486	93
5.67	288	98	1,388	100
6.25	282	100	1,428	97
6.65	320	88	1,628	85
6.99	384	73	2,046	68
7.60	1,002	28	3,120	45

Yeast invertase.*

pH	$n \times 10^5$	Proportional Nos.
3.25	587	86.0
4.02	654	95.9
4.59	681	99.8
4.88	682	100.0
5.32	678	99.4
5.67	654	95.9
6.67	474	69.5

*See Nelson and Bloomfield (26).

TABLE VI.

Activity and Hydrogen Ion Concentration.

Honey invertase preparation, "HD," data from curves in Fig. 2.

pH	Time for 10 per cent hydrolysis.	Proportional Nos.	Time for 35 to 55 per cent hydrolysis.	Proportional Nos.
	min.		*min.*	
4.15	310	35		
4.60	204	53	664	62
5.25	128	84	460	90
5.75	110	100	412	100
6.15	110	100	424	97
6.42	108	98	468	88
6.75	152	71	572	72

ion concentration in Figs. 6 and 7. They show that for honey invertase the range for optimum activity lies between hydrogen ion concentrations of $10^{-5.5}$ and $10^{-6.3}$.

This is quite different from the optimum range for yeast invertase which lies between hydrogen ion concentrations of $10^{-4.4}$ and $10^{-5.0}$. The honey invertase is most effective in a region considerably more alkaline than the optimum range for yeast invertase.

The Optimum Hydrogen Ion Concentration for Honey Invertase Differs for Different Stages of the Hydrolysis.—It was shown above

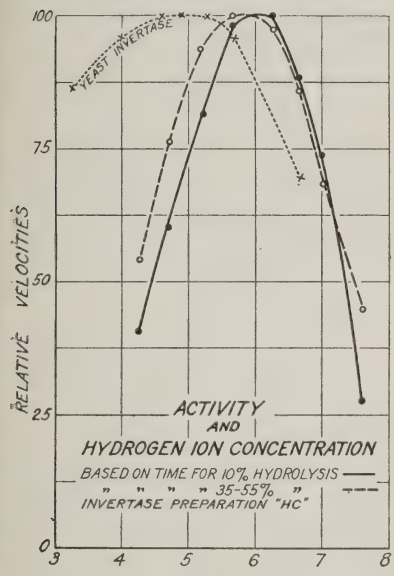

Fig. 6.

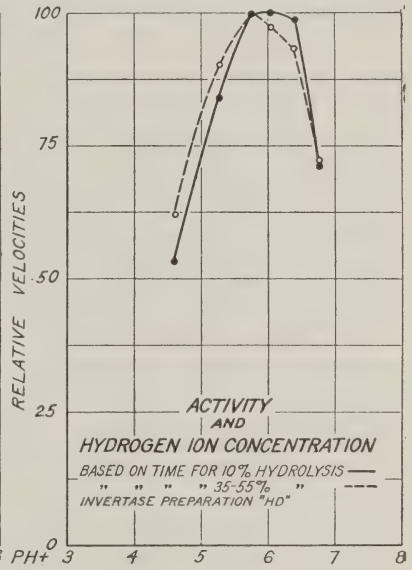

Fig. 7.

that hydrolyses of sucrose in the presence of honey invertase do not follow the same course at all hydrogen ion concentrations. It was, in fact, pointed out that some of the curves in Figs. 1 to 4, graphically representing the courses of inversions at various hydrogen ion concentrations, actually cross each other. Thus in the example used above, the hydrolysis at a hydrogen ion concentration of $10^{-6.25}$ (Fig. 1) at first proceeds at a faster rate than the inversion conducted at $10^{-5.67}$, but after about 20 per cent of the sugar present has been inverted the situation is re-

versed, the hydrolysis at $10^{-5.67}$ progressing faster than that at $10^{-6.25}$.

It then follows that if, instead of using the initial portions of the hydrolyses as the measure of the velocity, we use values based on the time consumed in the completion of some later portion, an entirely different result will be obtained for the optimum curve. This can be seen from the graphs in Fig. 6. The solid line curve in this figure represents the values obtained by plotting the relative initial velocities against hydrogen ion concentration. The curve drawn with a broken line is the result of plotting against hydrogen ion concentration, the relative velocities based on the time elapsed during the progress of the reaction from 35 to 55 per cent completion, in the same inversions from which the relative initial velocities were calculated. The position of this curve, based on the later stage of the inversions, is appreciably more to the acid side with respect to the hydrogen ion scale than is the curve based on initial velocities. Similar results were obtained with all the honey invertase preparations studied, but the extent of the difference in the optimum curves for different stages of the inversions does not appear to be exactly the same in all cases.

For yeast invertase hydrolyses, only one optimum curve is obtained, no matter what portion of the hydrolysis is taken as the criterion of the reaction velocity. This is to be expected from the fact, already mentioned, that the course of inversion in the presence of yeast invertase is unaffected by the hydrogen ion concentration of the substrate solution. It is interesting to note that the curve based on the rates of hydrolysis during the later portions of the inversions with honey invertase lies in an intermediate position on the hydrogen ion scale, between that occupied by the optimum curve for yeast invertase (dotted curve, Fig. 6) and that for honey invertase, based on initial velocities.

It further appears from the curves in Figs. 6 and 7 that the magnitude of the effect upon the rate of hydrolysis with honey invertase, due to hydrogen ion changes, is not the same at all stages of the hydrolysis. Moreover, the same relative change in hydrogen ion concentration seems to cause larger differences in the rate of inversion with honey invertase than with yeast invertase.

Stability of Honey Invertase Preparations and Hydrogen Ion Concentration.

When it was first noticed that the decline in velocity of hydrolysis in the later stages of inversions with honey invertase is greater in solutions alkaline to a hydrogen ion concentration of 10^{-6} than in more acid solutions, it was thought that this might be due to instability of the enzyme. Accordingly, inactivation tests were carried out (by a method described below) over a hydrogen ion range from $10^{-4.15}$ to $10^{-7.60}$. These tests showed that the enzyme is stable at hydrogen ion concentrations alkaline to 10^{-6}, and hence instability is not the cause of the rapid falling off of the reaction velocity in this region.

The inactivation tests were carried out in accordance with the method employed by Nelson and Hollander (27). To measured quantities of the invertase preparation, definite amounts of buffer were added to give the desired hydrogen ion concentration. With 1 part of the invertase buffer mixture a hydrolysis was immediately begun. The remainder of the mixture was allowed to incubate at 25°C. for 25 hours (in some cases the incubation period differed from this, as is noted in the data) and was used in an inversion begun the next morning.

The results, tabulated in Tables VII to IX, showed honey invertase to be stable in solutions alkaline to a hydrogen ion concentration of about 10^{-6}, but to grow progressively more unstable as the hydrogen ion concentration is increased above this point. This instability of honey invertase in the more acid region accounts for the rapidly diminishing reaction velocity of inversions conducted at hydrogen ion concentrations of $10^{-3.95}$, $10^{-4.15}$, and $10^{-4.25}$, which is noticeable in the curves representing these hydrolyses (Figs. 1 and 2).

In comparing the stability of honey and yeast invertases, it is found that yeast invertase can withstand greater acidities than can honey invertase. Nelson and Bloomfield (26) found no inactivation of the yeast invertase preparation they examined at 25°C., until a hydrogen ion concentration of $10^{-2.75}$ was reached; Nelson and Kerr (28) report some preparations which were partially unstable at a hydrogen ion concentration of $10^{-4.5}$. Both the honey and the yeast invertase appear to be stable in solutions alkaline to

TABLE VII.

Stability and Hydrogen Ion Concentration.

Honey invertase preparation, "HD."

10 cc. of invertase-buffer mixture (containing 8 cc. of invertase) in 225 cc. of solution.

Sucrose concentration : 10 gm. per 100 cc.

Temperature : 25°C.

Samples: 25 cc.

Na_2CO_3 (0.1M): 5 cc.

Polariscope tube : 400 mm.

pH	Time.	Observed rotation (I) (no incubation period).	Observed rotation (II) (25 hr. incubation period).	Difference between rotations I and II.
	min.			
4.15	0	25.91	25.91	
	40	25.61	25.68	0.07
	80	25.20	25.40	0.20
	120	24.74	25.07	0.33
	300	22.43	23.32	0.89
	525	19.44	20.94	1.50
4.60	0	25.92	25.92	
	40	25.41	25.49	0.08
	80	24.76	24.87	0.11
	120	23.98	24.16	0.18
	300	20.17	20.62	0.45
	525	15.99	16.68	0.69
5.25	0	25.91	25.91	
	40	24.97	25.04	0.07
	80	23.86	23.94	0.08
	120	22.55	22.68	0.13
	300	17.07	17.21	0.14
	525	12.31	12.50	0.19
	1,480	2.62	2.80	0.18
5.75	0	25.89	25.89	
	40	24.87	24.87	0.00
	80	23.44	23.50	0.06
	120	21.89	21.96	0.07
	300	15.88	15.97	0.09
	525	11.11	11.20	0.09
	1,435	2.05	2.14	0.09
6.42	0	25.90	25.90	
	40	24.82	24.84	0.02
	80	23.46	23.48	0.02
	120	22.03	22.05	0.02
	300	16.48	16.45	−0.03
	525	11.95	11.94	−0.01
	1,480	2.85	2.85	0.00

TABLE VII—*Concluded.*

pH	Time.	Observed rotation (I) (no incubation period).	Observed rotation (II) (25 hr. incubation period).	Difference between rotations I and II.
	min.			
6.78	0	25.91	25.91	
	40	25.03	25.00	−0.03
	80	23.92	23.91	−0.01
	120	22.70	22.68	−0.02
	300	17.79	17.75	−0.04
	525	13.47	13.45	−0.02
	1,435	4.56	4.53	−0.03

TABLE VIII.

Stability and Hydrogen Ion Concentration.

Honey invertase preparation, "HC."

10 cc. of invertase-buffer mixture (containing 5 cc. of invertase) in 225 cc. of solution.

Sucrose concentration: 10 gm. per 100 cc.

Temperature : 25°C.

Samples: 25 cc.

Na_2CO_3 (0.1 M) : 5 cc.

Polariscope tube : 400 mm.

pH	Time.	Observed rotation (I) (no incubation period).	Observed rotation (II) (50 hr. incubation period).	Difference between rotations I and II.
	min.			
4.25	0	25.90	25.89	
	$67\frac{1}{2}$	25.67	25.86	0.18
	135	25.41	25.73	0.31
	270	24.74	25.49	0.74
7.60	0	25.91	25.91	
	$67\frac{1}{2}$	25.74	25.76	0.02
	135	25.57	25.56	−0.01
	270	25.15	25.13	−0.02

their respective optima ($10^{-6.0}$ and $10^{-4.9}$), and unstable in more acid solutions.

Course of Hydrolysis and Sucrose Concentration.

In order to determine what changes in the course of reaction in honey invertase hydrolyses are induced by changes in the initial

sucrose concentration, several series of inversions were conducted in which the initial sucrose concentration was varied from 2 to 10 gm. per 100 cc., while the hydrogen ion concentration was kept constant (at $10^{-5.67}$ to $10^{-5.72}$ and $10^{-5.78}$ to $10^{-5.82}$) within the optimum range. The results of these experiments are recorded in Tables X to XII, and plotted in Figs. 8 and 9. From them it can be seen that the initial velocity of inversion increases with the increase of sucrose concentration until a point between 3 and 4 per cent is reached. Thereafter, a further increase in sucrose concentration is not accompanied by an increase in initial velocity.

TABLE IX.

Stability and Hydrogen Ion Concentration.

Honey invertase preparation, "HA."

20 cc. of invertase-buffer mixture, containing 10 cc. of invertase, 10 gm. of sucrose, and 80 cc. of H_2O.

Temperature: 25°C.
Samples: 25 cc.
Na_2CO_3 (0.1M): 5 cc.
Polariscope tube: 200 mm.

pH	Time.	Observed rotation (I) (no incubation period).	Observed rotation (II) (4 hr. incubation period).	Difference between rotations I and II.
	min.			
6.38	0	11.60	11.60	
	100	6.49	6.49	0.00
	200	3.97	3.95	−0.02
	400½	1.24	1.24	0.00

This characteristic of reaching an optimum sucrose concentration at about 4 per cent is held in common by the honey and yeast invertases (23). Yeast invertase hydrolyses also show increasing velocity with increase of sucrose concentration until a concentration of about 4 per cent is reached. Above this concentration the reaction velocity in the presence of yeast invertase does not increase with added increments of sucrose, just as is the case with honey invertase.

Under the heading, "Course of hydrolysis and hydrogen ion concentration," it was noted that in hydrolyses of 10 per cent sucrose solutions with honey invertase, an increase in reaction velocity—absent in yeast invertase hydrolyses—sets in soon after

TABLE X.

Rate of Hydrolysis and Sucrose Concentration.

5 cc. of honey invertase preparation, "HD," in 225 cc. of solution.
pH : 5.67 to 5.71.
Temperature: 25°C.
Samples: 25 cc.
Na_2CO_3 (0.1 M): 5 cc.
Polariscope tube: 400 mm.

Sucrose concentration.	Time.	Observed rotation.	Change in rotation.	Difference in successive readings.
gm. per 100 cc.	*min.*			
1	0	2.55		
	5	2.47	0.08	0.08
	10	2.40	0.15	0.07
	15	2.33	0.22	0.07
	30	2.10	0.45	0.23
	50	1.85	0.70	0.25
	90	1.37	1.18	0.48
	120	1.10	1.45	0.27
2	0	5.11		
	5	5.00	0.11	0.11
	10	4.90	0.21	0.10
	15	4.80	0.31	0.10
	30	4.49	0.62	0.31
	50	4.08	1.03	0.41
	90	3.31	1.80	0.77
	120	2.79	2.32	0.52
3	0	7.73		
	5	7.61	0.12	0.12
	10	7.48	0.25	0.13
	15	7.36	0.37	0.12
	30	6.98	0.75	0.38
	50	6.46	1.27	0.52
	90	5.44	2.29	1.02
	150	4.18	3.55	1.26
4	0	10.32		
	5	10.19	0.13	0.13
	10	10.06	0.26	0.13
	15	9.93	0.39	0.13
	30	9.55	0.77	0.38
	50	8.95	1.37	0.60
	90	7.83	2.49	1.12
	150	6.23	4.09	1.60

TABLE X—*Concluded.*

Sucrose concentration.	Time.	Observed rotation.	Change in rotation.	Difference in successive readings.
gm. per 100 cc.	*min.*			
6	0	15.53		
	5	15.40	0.13	0.13
	10	15.28	0.25	0.12
	15	15.15	0.38	0.13
	30	14.70	0.83	0.45
	50	14.02	1.51	0.68
	90	12.65	2.88	1.37
	150	10.58	4.95	2.07
10	0	25.92		
	5	25.81	0.11	0.11
	10	25.69	0.23	0.12
	15	25.56	0.36	0.13
	30	25.09	0.83	0.47
	50	24.44	1.48	0.65
	90	22.90	3.02	1.54
	150	20.40	5.52	2.50

TABLE XI.

Rate of Hydrolysis and Sucrose Concentration.

5 cc. of honey invertase preparation, "HC," in 225 cc. of solution.
pH: 5. 67 to 5.72.
Temperature: 25°C.
Samples: 25 cc.
Na_2CO_3 (0.1M): 5 cc.
Polariscope tube: 400 mm.

Sucrose concentration.	Time.	Observed rotation.	Change in rotation.	Difference in successive readings.
gm. per 100 cc.	*min.*			
2	0	5.13		
	30	4.93	0.20	0.20
	67½	4.71	0.42	0.22
	115	4.40	0.73	0.31
	190	3.90	1.23	0.50
	370	2.89	2.24	1.01
	1,895	0.63	4.50	2.26
4	0	10.33		
	30	10.11	0.22	0.22
	67½	9.78	0.55	0.33
	115	9.34	0.99	0.44
	190	8.62	1.71	0.72
	370	7.04	3.29	1.58
	1,895	3.05	7.28	3.99

TABLE XI—*Concluded.*

Sucrose concentration.	Time.	Observed rotation.	Change in rotation.	Difference in successive readings.
gm. per 100 cc.	*min.*			
6	0	15.51		
	30	15.28	0.23	0.23
	67½	14.95	0.56	0.33
	115	14.46	1.05	0.49
	190	13.61	1.90	0.85
	370	11.63	3.88	1.98
	1,895	6.34	9.17	5.29
10	0	45.91		
	67½	25.34	0.57	0.57
	135	24.57	1.34	0.77
	270	22.75	3.16	1.82
	540	19.26	6.65	3.49

TABLE XII.

Rate of Hydrolysis and Sucrose Concentration.

10 cc. of honey invertase preparation, "HB," in 100 cc. of solution.
pH: 5.78 to 5.82.
Temperature: 25°C.
Samples: 25 cc.
Na_2CO_3 (0.1 M) : 5 cc.
Polariscope tube: 400 mm.

Sucrose concentration.	Time.	Observed rotation.	Change in rotation.	Difference in successive readings.
gm. per 100 cc.	*min.*			
3	0	7.72		
	4	7.34	0.38	0.38
	8	6.94	0.78	0.40
	12	6.56	1.16	0.38
	20	5.75	1.97	0.81
4	0	10.33		
	4	9.97	0.36	0.36
	8	9.56	0.77	0.41
	16	8.70	1.63	0.86
	25	7.60	2.73	1.10
6	0	15.53		
	4	15.16	0.37	0.37
	8	14.74	0.79	0.42
	16	13.67	1.86	1.07
	25	12.44	3.09	1.23

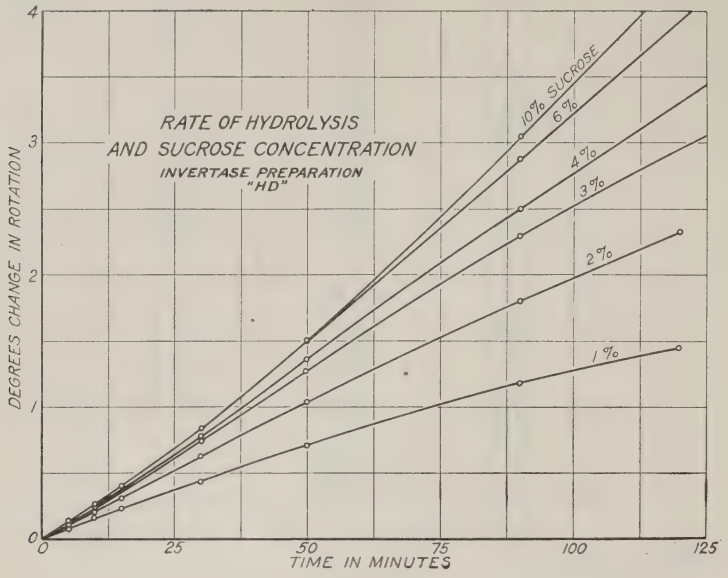
RATE OF HYDROLYSIS
AND SUCROSE CONCENTRATION
INVERTASE PREPARATION
"HD"
10% SUCROSE
6%
4%
3%
2%
1%
DEGREES CHANGE IN ROTATION
TIME IN MINUTES
0
25
50
75
100
125
1
2
3
4

Fig. 8.

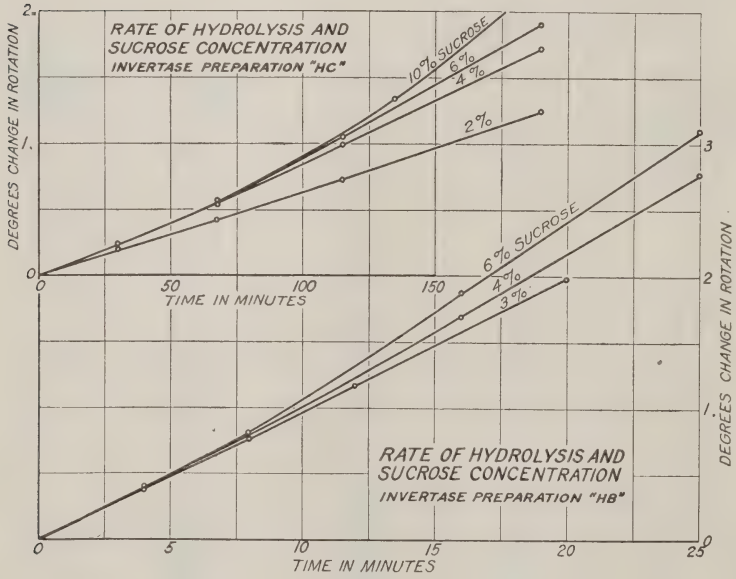
RATE OF HYDROLYSIS AND
SUCROSE CONCENTRATION
INVERTASE PREPARATION "HC"
10% SUCROSE
6%
4%
2%
DEGREES CHANGE IN ROTATION
TIME IN MINUTES
0
50
100
150
1
2
RATE OF HYDROLYSIS AND
SUCROSE CONCENTRATION
INVERTASE PREPARATION "HB"
6% SUCROSE
4%
3%
TIME IN MINUTES
0
5
10
15
20
25
1
2
3

Fig. 9.

the inversion is begun. The data and curves, given by the hydrolyses in which the sucrose concentration was varied (Tables X to XII, and Figs. 8 and 9), show that this increase in velocity took place in all those inversions in which the sucrose concentration was 4 per cent or over. Below this sucrose concentration honey invertase hydrolyses run more nearly like those with yeast invertase in that no increase in the velocity of hydrolysis sets in.

SUMMARY.

A method has been developed for obtaining an invertase from honey, in clear, transparent solutions.

The course followed by the hydrolysis of sucrose in the presence of the invertase from honey has been studied under several different conditions, such as varying hydrogen ion and sucrose concentrations.

The invertases from the two sources show several distinct differences in the way they cause the hydrolysis of sucrose to proceed.

The course of hydrolysis with honey invertase, in the case of sucrose concentrations of 4 per cent or higher, is marked by an increase in the inversion rate soon after the reaction begins. No such initial increase in velocity is found in the case of the hydrolysis with yeast invertase.

After the first portion of the hydrolysis is completed, the rate of inversion with honey invertase drops off relatively more rapidly than in the case of hydrolysis with yeast invertase.

The invertase from honey has an optimum activity range at hydrogen ion concentrations from $10^{-5.5}$ to $10^{-6.3}$, whereas the optimum range for the invertase from yeast lies between $10^{-4.4}$ and $10^{-5.0}$.

The optimum hydrogen ion concentration for the activity of honey invertase varies for different stages in the hydrolysis of sucrose, while in the case of yeast invertase it remains the same for all stages.

Both honey and yeast invertases are more stable in solutions somewhat alkaline to their respective optima than in more acid solutions. Yeast invertase can withstand greater acidities than honey invertase, some preparations of the former being stable at hydrogen ion concentrations of $10^{-3.0}$, whereas the honey

invertase preparations studied were partially inactivated at a hydrogen ion concentration of $10^{-5.8}$.

With increasing sucrose concentrations both honey and yeast invertases show increasing initial velocities of hydrolysis until an optimum sucrose concentration is reached. This optimum sucrose concentration is at about 4 gm. per 100 cc. in both instances.

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THE EFFECT OF HEAT TREATMENT OF MILK FEEDINGS ON THE MINERAL METABOLISM OF INFANTS.*

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It has been observed that young rats fed long heat-treated milks[1]—evaporated, condensed, and pasteurized by the "hold" process—fail to grow normally, the degree of stunting being proportional to the length of time of heating rather than to the temperature employed. For example, rats fed milk pasteurized by the "hold" process made only about half their normal growth; whereas, those fed milk pasteurized by the "flash" method, or quickly boiled, grew quite normally. Animals fed evaporated milk made scarcely any growth, while with those on the sweetened condensed milk, growth was comparable to that on milk pasteurized by the "hold" process. The nutritive failures in all cases appear to be due to the fact that during the process of heating, the calcium salts are thrown more or less out of solution and thus made less readily available to the animals, by adhering to the sides and bottom of the containers, or by more complete precipitation, most noticeable in evaporated milks. When care was taken to incorporate the precipitated calcium salts into the various milks by suspension in a starch paste, growth was normal. In the investigation there is nothing to indicate whether the results were due to a readjustment in the calcium, a change from the mono- and diphosphate to the triphosphate, or to a change in the colloid structure of the milk.[2]

It is conceivable that slight losses in the calcium content of cow's milk subjected to various methods of heat treatment may

* This study was made possible through the courtesy of the Department of Pediatrics, College of Medicine, State University of Iowa.

[1] Daniels, A. L., and Loughlin, R., *J. Biol. Chem.*, 1920, xliv, 381.

[2] Palmer, L. S., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 137.

have considerable influence on the nutritive condition of rats fed exclusively thereon, for their calcium requirement is relatively high; but that such losses should have a practical bearing on the artificial feeding of infants did not at first seem probable since the customary milk mixtures used in infants' feedings are considerably higher in calcium than is human milk. However, a study of the weight charts of a number of well babies in our clinic suggested that the method of "sterilizing" milk feedings may have a direct influence on the physiologic well-being of the baby. The particular babies under observation appeared normal in every way, although they were not gaining on pasteurized milk feeding mixtures which furnished considerably more food than is often considered necessary, and were on formulas which we had every reason to believe furnished enough of the various essential constituents. The feedings, which were carefully prepared by one of us, consisted of Holstein milk equal to 10 per cent or more of the body weight of the baby[3], cream in some cases, a carbohydrate addition, Dextri-Maltose or lactose, and boiled water. The usual $\frac{1}{2}$ ounce of orange juice was given daily, thereby ruling out the possible influence of the destruction of the antiscorbutic vitamin in the various mixtures. In a certain number of cases when the method of sterilizing the milk mixtures was changed from pasteurized to quickly boiled, growth which had hitherto been stationary, was at once resumed. The case of J. W. (Chart 1) is particularly noteworthy. On the same feeding mixture this baby's weight during two pasteurized periods remained stationary, while on the two boiled milk periods there was a marked increase in weight.

Although there is a considerable evidence pointing to the conclusion that the antineuritic vitamin as found in foods is thermostable[4] at least up to 120°C., it is possible that some slight destruction may take place even at pasteurization temperatures, and in a mixture containing the vitamin in low concentration[5] this would be evidenced by a stationary weight. Quick boiling

[3] Theoretical weight, after Finkelstein = birth weight in grams + (age in months × 600) − 300 for first 6 months; birth weight + (age in months × 500) for the second 6 months.

[4] Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, 1920, xliii, 265.

[5] Daniels, A. L., Byfield, A. H., and Loughlin, R., *Am. J. Dis Child.*, 1919, xviii, 546.

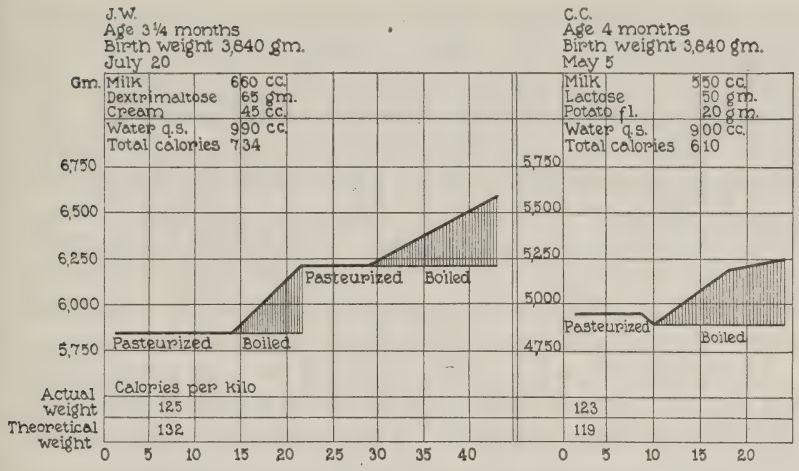

Chart 1. Comparison of growth curves of babies fed milk mixtures pasteurized by the "hold" process during one period, and quickly boiled during a subsequent period.

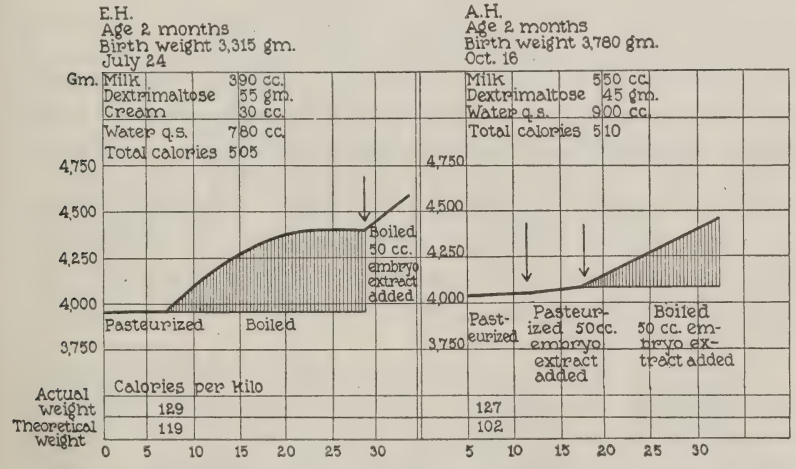

Chart 2. The addition of wheat embryo extract to boiled milk feedings stimulated growth in the case of E. H.; whereas, the addition of this to the pasteurized feedings in the case of A. H. resulted in only a slight gain in weight. When this mixture was subsequently boiled there was a marked increase in weight.

may produce less change in the vitamin that the "hold" method of pasteurization. The weight increases, however, during the boiled milk periods, would seem to be the results of some factor other than the possible larger amount of the antineuritic vitamin of the boiled milk. The addition of 50 cc. of our water-alcoholic extract of wheat embryo (known to contain the antineuritic vitamin) to the pasteurized feedings of Baby A. H. (Chart 2) caused only a very slight increase in weight. When, subsequently, this milk mixture was boiled, there was a marked increase in weight, the result, apparently, of the combined influence of the shorter time of heating and the antineuritic vitamin addition.

Changes in the method of heat-treating the milk feedings have not resulted in growth stimulation in all our babies. It is possible that those children who failed to gain were receiving enough of the essential inorganic constituents, and the additional amount made available by the shorter heating was without influence; or there may have been other factors as yet unappreciated, concerned in the growth stimulation.

So far as we have been able to find the literature contains no data concerning the availability of the mineral constituents of milk "sterilized" by different methods. Söldner[6] early pointed out that the lime salts in heated milk are not only useless for rennet coagulation but are also not suitable for absorption. Arndt[7] and Cronheim and Müller[8] studied the metabolism of calcium in infants on fresh and boiled milk. The results are not conclusive. Many comparative clinical studies of children fed milk, raw and boiled, and raw and pasteurized, have also been reported, but these were not sufficiently controlled to be of more than general interest.

The prevalence of rickets and the generally accepted hypothesis that rickets is the result of a faulty calcium and phosphorus metabolism led us to investigate the availability of the inorganic salts, more particularly the calcium and phosphorus in milk

[6] Söldner, F., Die Salz der Milch und ihre Beziehungen zu dem Verhalten des Kasein, Dissertation, Erlangen, 1888.

[7] Arndt, J., Das Verhalten der Kalksalze in den Faeces und im Harn von Säuglingen bei Darreichung gekochter und ungekochter Milch, Inaugural dissertation, Breslau, 1901.

[8] Cronheim, W., and Müller, E., *Jahrb. Kinderh.*, 1903, lvii, 45.

feedings "sterilized" by the two more usual methods of heating, and to determine whether the growth stimulation in our babies following the change from pasteurized to quickly boiled feedings might not be in part, at least, the result of better calcium utilization.

In the investigation, mineral balance studies were made on normal boy babies varying in age from 3 to 7 months. And because previous experience had taught us that collections made by the busy undergraduate nurse could not be relied upon, during the period of study the babies were cared for day and night in the metabolism ward by graduate nurses trained in metabolism ward technique. These were on 8 hour shifts.

Especial care was taken in feeding the babies so that there were no refusals or food losses. By special adaptation of the Hoobler metabolism frame, stools and urine were collected quantitatively during 3 day periods. The fecal periods were marked off with charcoal prepared from cane-sugar. During some preliminary work, when the stools were not marked off, it was observed that the daily variations in the fecal calcium, phosphorus, and nitrogen were so great as to make us question the constancy of daily elimination;[9] therefore, the procedure of marking off the stools for the 3 day period was adopted. With this method the differences in daily excretion were found to be relatively slight. As soon as the stools

[9] An example of the variation in the daily fecal elimination of calcium when stools were not marked off is shown in the following table:

Baby W.

Milk.	Day.	Dry weight of stool.	CaO
		gm.	*gm.*
Pasteurized.	1	8.508	0.6982
	2	8.060	0.5660
	3	10.680	0.7042
Boiled.	1	8.996	0.6024
	2	8.299	0.5368
	3	10.170	0.6724

Such results may not be averaged, and in the work reported have not been included because they seemed unreliable.

were passed they were thoroughly mixed with acid alcohol, dried on the water bath, pulverized, dried to constant weight, and an aliquot was taken for analysis. The urines were collected under toluene. Occasionally a "leak" occurred. In such instances, the collection for the day was discarded and a new period started. Creatinine determinations were made on each 24 hour specimen in order to check the completenesss of the collections. These creatinine figures were found to be constant, varying only from 1 to 5 mg. from day to day. In the tables the average for the 3 day period is given.

Several days before the babies were put on the frame they were given the particular milk mixture to be tested. In all cases during the first period of a series, the milk feedings were pasteurized. In the second period, begun immediately after the close of the first period, the feedings were quickly boiled, no other change being made. Although the babies seemed perfectly contented and happy on the frame at all times, they were allowed a bed rest of 3 or 4 days before being returned to the frame for a second period. With a few of the older babies double periods were run. It is unfortunate that this was not possible in every case, but with many babies, especially the younger ones, it is not comfortable to hold them on a given milk mixture longer than 2 weeks.

The milk feedings were prepared with laboratory accuracy. Distilled water, free from all traces of calcium and magnesium, was used in making up the feedings and in diluting the daily dose of orange juice ($\frac{1}{2}$ ounce). Those mixtures to be pasteurized were distributed evenly in the requisite number of bottles (one for each feeding) and subsequently pasteurized at 145°F. for 30 minutes. With the boiled feedings, the ingredients were mixed in an open aluminum pan, brought quickly to the boiling temperature over a gas flame, and allowed to "roll up" three times, the entire process taking approximately 8 minutes. The feeding mixtures were then poured into the required number of sterile bottles. In both cases the filled bottles were cooled as quickly as possible in running water and kept on ice until needed. The samples of feeding mixtures taken for analysis were prepared similarly, an aliquot of the 24 hour mixture being used.

Since our chief interest in the investigation was concerned with the difference in the availability of the calcium and phosphorus

TABLE I.

Calcium (CaO) Retention in Infants Fed Pasteurized and Boiled Milk Mixtures.

Case.	Age.	Weight.	Milk period.	CaO excretion (3 days).			CaO intake.		CaO retention.		
				Urine.	Feces.	Total.	3 days.	Daily per kg.	Total.	Daily per kg.	Per cent of intake.
	mos.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
M. I.	5	5,550	Pasteurized.	0.085	2.951	3.026	3.556	0.213	0.530	0.031	14.94
		6,200	Boiled.	0.098	2.711	2.809	3.556	0.191	0.747	0.040	21.02
W. A.	6	6,400	Pasteurized.	0.148	3.209	3.357	3.556	0.185	0.199	0.010	5.66
		6,500	Boiled.	0.121	2.915	3.036	3.556	0.182	0.520	0.026	14.69
G. V.	3	4,450	Pasteurized.	0.102	2.685	2.787	2.571	0.192	−0.216	−0.016	−8.41
		4,620	Boiled.	0.069	2.307	2.376	2.571	0.185	0.195	0.014	7.60
M. P.	7	6,925	Pasteurized.	0.112	3.870	3.982	3.804	0.183	−0.178	−0.008	−4.64
		7,000	Boiled.	0.087	3.811	3.898	3.804	0.181	−0.094	−0.004	−2.43
		7,175	Pasteurized.	0.105	3.769	3.874	4.044	0.188	0.170	0.007	4.23
		7,350	Boiled.	0.099	3.761	3.860	4.044	0.183	0.184	0.008	4.54
G. G.	7	6,050	Pasteurized.	0.184	3.135	3.319	3.552	0.196	0.233	0.013	6.66
		6,400	Boiled.	0.062	2.611	2.673	3.552	0.185	0.879	0.045	24 72
		6,550	Pasteurized.	0.081	3.221	3.302	3.792	0.193	0.490	0.025	12.90
		6,700	Boiled.	0.094	2.592	2.686	3.792	0.189	1.106	0.055	29.20
D. W.	6	5,765	Pasteurized.	0.107	4.389	4.496	4.424	0.260	−0.072	−0.004	−1.60
		5,950	Boiled.	0.108	3.795	3.903	4.424	0.248	0.521	0.029	11.88
		6,225	Pasteurized.	0.108	4.422	4.530	4.424	0.237	−0.106	−0.005	−2.46
		6,475	Boiled.	0.106	3.848	3.954	4.424	0.228	0.570	0.024	10.62

on the different types of heat-treated milks, only those data are reported which point to this end; namely, the calcium and phosphorus content of the milk feedings, urines, and feces, the nitrogen balances, and the urinary creatinines.

The calcium determinations were made according to the method of McCrudden,[10] excepting in the case of the urines. The calcium content of infants' urine is so low that it was found that more uniform results were obtained when the urines were evaporated to dryness with nitric and hydrochloric acids to remove the organic matter, and analyses were made on the residues. 25 cc. of the sodium acetate solution were used in all cases instead of the 10 to 15 cc. recommended. Phosphates[11] (as P_2O_5) were determined as the phosphomolybdate and the final precipitate was weighed as magnesium pyrophosphate. Creatinine determinations were made according to the micro method of Folin[12] and total nitrogen by the Kjeldahl-Gunning method.

Either duplicate or triplicate determinations were made in all cases, depending upon the amount of material. When there was any question concerning a given result, second duplicate determinations were made. The figures reported are the checks, not the averages, of the various analyses.

The results of the investigation are summarized in Tables I to IV.

The most significant findings in the investigation are the conspicuously greater calcium retention in many of the babies during the boiled milk periods. In one case, Baby D. W., who was in negative calcium balance during both pasteurized milk periods, retained over eight times as much calcium during the first boiled milk period, and in the second series over six times as much calcium, as in the corresponding pasteurized milk periods. Had this baby been continued on pasteurized feedings, he would undoubtedly have developed rickets, although at the time the baby was being studied there were no apparent rachitic symptoms. The results with Baby G. G. were only slightly less striking, from two to three times as much calcium oxide being retained

[10] McCrudden, F. H., *J. Biol. Chem.*, 1909–10, vii, 83.

[11] Hawk, P. B., Practical physiological chemistry, Philadelphia, 8th edition, 1923, 581.

[12] Folin, O., *J. Biol. Chem.*, 1914, xvii, 469.

on the boiled feedings. It would seem from these results that the calcium in milk pasteurized by the "hold" method is less readily available than it is in quickly boiled milk.

TABLE II.

Phosphorus (P_2O_5) Retention in Infants Fed Pasteurized and Boiled Milk Mixtures.

Case.	Milk period.	P_2O_5 excretion (3 days).			P_2O_5 intake.		P_2O_5 retention.		
		Urine.	Feces.	Total.	3 days.	Daily per kg.	Total.	Daily per kg.	Per cent of intake.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
M. I.	Pasteurized.	2.450	1.570	4.020	4.498	0.270	0.478	0.028	10.61
	Boiled.	2.391	1.440	3.831	4.498	0.241	0.666	0.035	14.81
W. A.	Pasteurized.	2.368	1.740	4.108	4.498	0.234	0.390	0.020	8.87
	Boiled.	2.038	1.973	4.011	4.498	0.231	0.487	0.025	10.81
G. V.	Pasteurized.	1.429	1.773	3.202	3.435	0.257	0.233	0.017	6.80
	Boiled.	1.551	1.473	3.024	3.435	0.248	0.411	0.029	11.93
M. P.	Pasteurized.	2.878	2.608	5.476	5.298	0.255	−0.178	−0.008	−3.33
	Boiled.	2.761	2.569	5.330	5.298	0.252	−0.032	−0.001	−0.63
	Pasteurized.	3.181	1.987	5.168	5.544	0.258	0.376	0.017	6.86
	Boiled.	2.959	2.058	5.017	5.544	0.251	0.527	0.024	9.50
G. G.	Pasteurized.	2.529	2.073	4.602	4.884	0.269	0.282	0.015	5.81
	Boiled.	2.338	2.054	4.392	4.884	0.254	0.492	0.025	10.10
	Pasteurized.	2.865	1.909	4.774	5.184	0.264	0.410	0.021	7.90
	Boiled.	2.117	1.871	3.988	5.184	0.258	1.196	0.059	23.10
D. W.	Pasteurized.	2.076	3.088	5.164	5.559	0.326	0.395	0.023	7.10
	Boiled.	2.422	2.543	4.965	5.559	0.311	0.594	0.033	10.70
	Pasteurized.	2.639	2.519	5.158	5.559	0.298	0.401	0.021	7.21
	Boiled.	2.457	2.525	4.982	5.559	0.286	0.577	0.029	10.30

It is well known that the greater part of the calcium eliminated from the body is by way of the tract; nevertheless, if the different methods of heat-treating the milk mixtures results in a change in the form of the calcium contained therein, thereby making it less available, we should expect that this would be made manifest by a somewhat greater output in the stools of the babies

when they were receiving the longer heat-treated milk. Such was found to be the case. With two of the babies on the double periods (G. G. and D. W.) it will be observed that the fecal calcium was practically the same on the two pasteurized milk periods,

TABLE III.

Nitrogen Retention in Infants Fed Pasteurized and Boiled Milk Mixtures.

Case.	Milk period.	Creatinine 3 days.	N excretion (3 days).			N intake.		N retention.		
			Urine.	Feces.	Total.	3 days.	Daily per kg.	Total.	Daily per kg.	Per cent of intake.
		mg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
M. I.	Pasteurized.	251	7.74			10.62				
	Boiled.	258	6.26	1.41	7.67	10.62	0.571	2.95	0.158	27.7
W. A.	Pasteurized.	245	7.14	1.02	8.16	10.62	0.553	2.46	0.128	23.2
	Boiled.	241	6.64	1.34	7.98	10.62	0.545	2.64	0.135	24.8
G. V.	Pasteurized.	174	4.64	0.84	5.48	7.65	0.573	2.17	0.162	28.3
	Boiled.	171	4.21	0.69	4.90	7.65	0.552	2.75	0.199	36.0
M. P.	Pasteurized.	233	10.06	1.24	11.30	12.48	0.601	1.18	0.056	9.4
	Boiled.	228	8.49	1.27	9.76	12.48	0.595	2.72	0.130	21.8
	Pasteurized.	236	9.20	1.14	10.34	12.99	0.603	2.65	0.123	20.4
	Boiled.	229	8.18	1.18	9.36	12.99	0.589	3.63	0.165	28.0
G. G.	Pasteurized.	233	7.19	1.02	8.21	10.92	0.602	2.71	0.149	24.7
	Boiled.	232	7.21	1.04	8.25	10.92	0.569	2.67	0.139	24.4
	Pasteurized.	229	8.27	1.20	9.47	11.68	0.594	2.21	0.113	19.0
	Boiled.	234	7.97	1.30	9.27	11.68	0.581	2.41	0.119	20.5
D. W.	Pasteurized.	236	8.50	1.91	10.41	13.71	0.805	3.30	0.194	24.1
	Boiled.	232	9.85	1.55	11.40	13.71	0.768	2.31	0.129	16.8
	Pasteurized.	235	10.47	1.37	11.84	13.71	0.734	1.87	0.099	13.5
	Boiled.	235	10.09	1.23	11.32	13.71	0.706	2.39	0.124	17.6

and within the limits of experimental error on the two boiled milk periods. It seems reasonable to suppose that the differences in the amount of calcium lost through the bowel on the two types of heat-treated milk feedings are the result of changes incidental to the heating processes.

Very little is known positively concerning the conditions which determine the excretion of calcium through the kidneys. The urinary calcium in our series did not run parallel with the fecal calcium. In some cases it was considerably higher on the pasteurized milk feedings; in other cases, it was the same or slightly lower. Were it not for our creatinine values we would be led to believe that these variations were due to incomplete urine collections. As it is, we are unable to offer any explanations for the seemingly discordant results.

The total phosphorus retention on the two types of heat-treated milks was fairly consistent with the calcium retentions, and in all cases was considerably greater during the boiled milk feedings. The relationship, however, between the fecal phosphorus during the two types of milk feedings is not so marked as in the case of the fecal calcium. In some instances there was slightly more phosphorus in the stool during the pasteurized milk feedings, in others the fecal phosphorus was the same, or slightly greater on the boiled milk periods. The urinary phosphorus, on the other hand, was in general higher during the pasteurized milk periods. The increased phosphorus retention in our babies appears to be due to a metabolic adjustment rather than to a greater absorption.

The data relative to the nitrogen excretion have been included in the report to show, first, that our babies were getting a liberal allowance of protein, and second, that there appears to be some relationship between the calcium, phosphorus, and nitrogen retentions. It will be noted that in all but two cases there was an increased nitrogen retention during the boiled milk period. The conspicuously greater retention in Baby D. W. during the first pasteurized milk period may possibly be explained by the fact that the baby was slightly underfed during the preliminary experimental period although we did not appreciate it at the time. During the 1st night on the frame it was observed that the baby at about 2.00 a.m. seemed very restless and hungry. The nurse in charge, knowing that the baby must be held on the given formula for 2 or 3 weeks, gave him a 2 o'clock bottle, which practice was continued throughout the observation periods. This sudden increase of food, although disturbing the nitrogen retention sequence in the first period, did not appear to alter the calcium and phosphorus relationships.

TABLE IV.

Comparison of Daily Retention of Calcium, Phosphorus, and Nitrogen during Pasteurized and Boiled Milk Periods.

Case.	Milk period.	CaO	P_2O_5	Nitrogen.	Feeding mixtures.
		gm.	*gm.*	*gm.*	
M. I.	Pasteurized.	0.177	0.159		Milk, 700 cc.; Dextri-maltose, 75 gm.
	Boiled.	0.249	0.222	0.98	Water, q.s. 1,050; 7 × 150 cc.
W. A.	Pasteurized.	0.066	0.130	0.82	Milk, 700 cc.; Dextri-maltose, 75 gm.
	Boiled.	0.173	0.162	0.88	Water, q.s. 960; 6 × 160 cc.
G. V.	Pasteurized.	−0.072	0.078	0.72	Milk, 480 cc.; Dextri-maltose, 45 gm.
	Boiled.	0.065	0.137	0.92	Water, q.s. 840; 6 × 140 cc.
M. P.	Pasteurized.	−0.059	−0.059	0.39	Milk, 750 cc.; Dextri-maltose, 45 gm.
	Boiled.	−0.031	−0.011	0.91	Lactose, 30 gm.; water, q.s. 1,060; 6 × 170 cc.
	Pasteurized.	0.057	0.125	0.88	Milk, 700 cc.; skim milk, 100 cc.; Dextri-maltose, 45 gm.
	Boiled.	0.061	0.175	1.21	Lactose, 30 gm.; water, q.s. 1,020; 6 × 170 cc.
G. G.	Pasteurized.	0.078	0.094	0.90	Milk, 700 cc.; Dextri-maltose, 45 gm.
	Boiled.	0.293	0.164	0.89	Lactose, 25 gm.; water, q.s. 960; 6 × 160 cc.
	Pasteurized.	0.163	0.137	0.74	Milk, 600 cc.; skim milk, 150 cc.; Dextri-maltose, 45 gm.
	Boiled.	0.369	0.399	0.80	Lactose, 30 gm.; water, q.s. 960; 6 × 160 cc.
D. W.	Pasteurized.	−0.024	0.132	1.10	Milk, 700 cc.; skim milk, 116 cc.
	Boiled.	0.174	0.198	0.77	Lactose, 45 gm.; Dextri-maltose, 23 gm.
	Pasteurized.	−0.035	0.134	0.62	Water, q.s. 1,155; 7 × 165 cc.
	Boiled.	0.157	0.192	0.80	

The fecal nitrogen was not consistently greater during the pasteurized milk periods. In many cases it was the same in both periods. The urinary nitrogen, on the other hand, was in

general higher during the pasteurized milk period. It would seem, therefore, that the pasteurization process did not affect the availability of the protein of the milk, but that the greater retention of nitrogen during the boiled milk periods was related to the calcium and phosphorus retentions. Furthermore, it is probable that the increase in weight in those babies who were holding on pasteurized milk feedings and gained when the mixtures were subsequently boiled, was due to the greater nitrogen retention. More data, however, on this point are needed before conclusions can be drawn.

A comparison of the data relative to the calcium and phosphorus retention in our babies with those of certain other investigators suggests that our babies even under the most favorable conditions, during the boiled milk periods, were retaining considerably less than what is generally considered the optimum amount of calcium. Holt, Courtney, and Fales[13] observed that the average absorption of calcium by breast-fed babies was 0.06 gm. per kilo, and of those fed cow's milk, 0.09 gm. of calcium oxide per kilo of body weight; whereas, our infants were retaining only from 0.008 to 0.055 gm. per kilo with an average of 0.030 on the boiled milk periods, and considerably less on the pasteurized milk periods, in spite of the fact that an adequate amount of fat (4 gm. per kilo[13]) was being ingested. It should be noted, however, that the majority of the so called normal babies reported by Holt, Courtney, and Fales were considerably underweight. Whereas, ours had gained regularly from birth and were up to the generally accepted standard of weight for their ages. They had no apparent sign of rickets or other physical stigma. It is possible that the lower retention in our babies was due to the fact that they were more nearly "full" of the various essential constituents and thus the retention was less than that found by other workers. On the other hand, it is probable that the calcium requirements of a baby are, in part, determined by its skeletal development. An underweight baby may have the same calcium needs as a normal baby of the same age. A study of the data given by the above mentioned authors from the standpoint of the theoretical weights of the babies, suggests that the calcium requirement of

[13] Holt, L. E., Courtney, A. M., and Fales, H. L., *Am. J. Dis. Child.*, 1920, xix, 97.

infants on cow's milk is more nearly in accord with the requirements as given for the breast-fed baby, namely 0.06 gm. per kilo; but even this figure is considerably higher than that found by certain other investigators. The breast-fed babies reported by Czerny and Keller[14] on the basis of their theoretical weights were retaining an average of 0.027 gm. of calcium oxide per kilo; whereas, the artifically fed babies reported by these authors were retaining an average of 0.031 gm. per kilo. Lindberg[15] reports a daily calcium oxide retention of 0.056 and 0.063 gm., respectively, in a $2\frac{1}{2}$ months' breast-fed infant, during two successive 3 day periods. On the basis of its theoretical weight this baby was retaining 0.012 and 0.038 gm. per kilo per day in the two periods. Tobler and Noll[16] also found what seemed to be a low calcium oxide retention. A $2\frac{1}{2}$ months' old breast-fed baby was retaining 0.053 gm. of calcium oxide or 0.0115 gm. per kilo, estimated on its theoretical weight. These findings are more nearly in accord with those we obtained in our 3 months' old artificially fed baby (G. V.), who during the boiled milk period was retaining 0.014 gm. of calcium oxide.

Schloss,[17] on the other hand, working with older rachitic infants, found in one case a daily calcium oxide retention of 0.024 gm. per kilo (theoretical weight) under most favorable conditions, when cod liver oil was given; and in a second case, a daily calcium retention of 0.013, 0.027, and 0.057 gm., respectively, on three successive cod liver oil periods in which the second contained phosphorized cod liver oil. This child was in negative calcium balance and presumably was depleted when the cod liver oil was added to the food. He probably was retaining somewhat more than the average amount of calcium during the cod liver oil periods, for Schabad[18] has shown that when convalescence or cure in rickets begins there is a greatly increased retention of calcium, which shows itself earlier than does clinical improvement. Besides

[14] Czerny, A., and Keller, A., Des Kindes Ernährung, Ernährungsstörungen und Ernährungstherapie, Leipsic and Vienna, 1917, ii, *9. Abt.*, 491.

[15] Lindberg, G., *Z. Kinderh., Orig.*, 1917, xvi, 90.

[16] Tobler, L., and Noll, F., *Monatschr. Kinderh.*, 1910, ix, 210.

[17] Frank, L., and Schloss, E., *Jahrb. Kinderh.*, 1914, lxxix, 539.

[18] Schabad, J. A., *Arch. Kinderh.*, 1910, liii, 380.

the factor of underweight in the babies reported by Holt, Courtney, and Fales, there may have been this added factor, depletion, and therefore the larger calcium oxide retention.

A comparison of the phosphorus retention in our babies on boiled milk mixtures with that of breast-fed infants suggests that in most instances our babies were retaining fairly liberal amounts of phosphorus. Tobler and Noll[16] report a case of a $2\frac{1}{2}$ months' old breast-fed baby who was retaining 0.119 gm. or 0.023 gm. per kilo on its theoretical weight, and Lindberg[15] found in two successive periods a $2\frac{1}{2}$ months' old breast-fed baby retained 0.04 and 0.03 gm. per kilo, respectively. These figures are in accord with the findings in our 3 months' old baby who was retaining 0.162 or 0.029 gm. of phosphorus per kilo. Hoobler,[19] on the other hand, obtained a phosphorus retention of 0.093 or 0.015 gm. per kilo in a 5 months' old breast-fed baby; whereas, our 5 months' old baby (M.I.) was retaining, on quickly boiled milk, 0.035 gm. per kilo.

What the optimum amount of calcium and phosphorus, infants of different ages should retain, is apparently unknown. Such information can only be obtained through studies of many babies under various conditions. The seemingly lower retentions in some of our cases may have been due to the fact that these babies were more nearly "full" of the various essentials; and thus the total retentions were less than those reported by certain other workers. Or it may be that the types of artificial feedings used were not such as to best meet the nutritive requirements of infants. Some more recent investigations which will be reported in the near future suggest that this may be the case. It is also possible that the difference in the method of collecting the stools explains some of the apparent discrepancies in the literature. The point we wish to make, however, is that the method of heat-treating the milk materially influences the availability of the calcium and phosphorus of the feeding mixture. Pasteurization may be the best method of making milk more nearly safe for infant feeding, although this has recently been questioned, but such heat-treated milk does not seem to be the most satisfactory from the standpoint of fulfilling the physiologic needs of the baby.

[19] Hoobler, B. R., *Am. J. Dis. Child.*, 1911, ii, 107.

SUMMARY.

1. In certain instances babies, who were holding in weight on given milk mixtures when pasteurized by the "hold" method, gained when the mixtures were quickly boiled, no other change being made.

2. Attention is called to the fact that in making calcium balance in infants, it is essential that stools be carefully marked off. When this was done it was found that the fecal elimination on the same diet was very constant.

3. The calcium and phosphorus retention in infants fed quickly boiled milk mixtures was considerably greater than it was when the milk mixtures were pasteurized.

4. The fecal calcium and phosphorus were greater during the period when pasteurized milk feedings were given, indicating that the longer heat treatment of milk results in a decrease in the availability of the phosphorus and calcium in the milk mixtures.

5. There appears to be some relationship between the calcium, phosphorus, and nitrogen retentions.

6. It seems probable that the calcium needs of children are very largely determined by their skeletal development. In determining the calcium needs of normal children it would seem more nearly fitting to include only those who are up to standard in weight, or to determine the calcium needs on the basis of the theoretical weights of the infants.

7. It is probable that a baby fed pasteurized milk over a long period of time is receiving too little calcium for his growth needs.

SOME EFFECTS OF INSULIN ON THE CARBOHYDRATE AND PHOSPHORUS METABOLISM OF NORMAL INDIVIDUALS.*

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The normal organism is able to effect the conversion of glucose into lactic acid or the reverse as indicated below.

(1) Glucose $\leftrightarrows$ lactic acid

This reaction is without doubt fundamentally concerned in carbohydrate metabolism.

Embden and coworkers have presented evidence for the existence in striated muscle of a compound of hexose with phosphoric acid. This substance is easily split into lactic acid and phosphoric acid by an enzyme present in muscle press-juice. Under favorable conditions these two acids are liberated in approximately equimolecular amounts. These authors have prepared from striated muscle a substance which yielded an osazone identical with that of hexose phosphoric acid previously isolated from yeast by Lebedew, and Young. Embden has named this substance "lactacidogen." It is believed to be of the utmost importance in the contractile process of muscle. A discussion of this subject is found in the recent paper by Himwich, Loebel, and Barr (1). Hexose diphosphate then might be considered an intermediary between glycogen and lactic acid as shown in reaction (2).

(2) Glycogen $\longrightarrow$ hexose diphosphate $\longrightarrow$ lactic acid

or the reverse

(3) Lactic acid $\longrightarrow$ hexose diphosphate $\longrightarrow$ glycogen

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We may therefore write reaction (1) as below.

(4) Glucose → lactic acid → hexose diphosphate → glycogen

or

(5) Glycogen → hexose diphosphate → lactic acid → glucose

The participation of P in this process is indicated in the following reaction.

(6) Lactic acid + Inorganic P → hexose diphosphate → glycogen + inorganic P

or

(7) Glycogen + Inorganic P → hexose diphosphate → lactic acid + inorganic P

If this combination of hexose with phosphoric acid is formed in the tissues other than the blood, then inorganic P should be withdrawn from the blood during the anabolic stage of carbohydrate metabolism and should reappear in this tissue when this compound has been broken up or when the reverse process is taking place. Changes in the content of inorganic P in the blood should be reflected in the excretion of P in the urine.

Fiske (2, 3) showed that the rate of excretion of P in the urine of a fasting individual falls during the early forenoon. If sucrose was given while the rate was falling there was a still greater decrease and if it was given when the curve was rising there was a lessening of the excretion rate. Salvesen (4) observed that the inorganic P of the blood serum of dogs decreases after the ingestion of glucose. Perlzweig, Latham, and Keefer (5) found that the ingestion of 50 to 100 gm. of glucose and the intravenous injection of 3.5 units of insulin, also the subcutaneous injection of 0.5 cc. of 1:1,000 solution of epinephrine caused in normal individuals a marked fall in the inorganic P of the blood serum and a simultaneous decrease in the rate of excretion of phosphate in the urine. The excretion of P was most singularly depressed by the ingestion of glycerol. While in all the cases studied this retention of P was accompanied by a rise of the respiratory quotient indicating increased metabolism of carbohydrate, with the ingestion of

glycerol the marked retention of P was accompanied by a lowered respiratory quotient. No relationship was found between the retention of P and the titratable acidity of the urine. Evidence of a hexose phosphate combination in the blood could not be found by hydrolyzing blood filtrates and then determining the reducing sugar. These authors conclude that if such compounds are formed they are to be found in the liver and in the muscles. Wigglesworth, Woodrow, Smith, and Winter (6) have also observed that insulin causes a drop in the inorganic P of the whole blood of rabbits. Briggs, Koechig, Doisy, and Weber (7) have recently reported that in dogs at the time of the hypoglycemia following insulin there is a decrease in the inorganic P and K and an increase in the lactic acid of the blood. These authors feel that their observations without doubt indicate that the disappearance of sugar is due to the formation of lactic acid, and they believe that insulin influences the reversible reaction,

$$\text{Glucose} \leftrightarrows \text{lactic acid}$$

in the direction of lactic acid and that glucose is formed in diabetic animals because this influence is lacking. Harrop and Benedict (8), in a recent paper, report experiments which show that insulin causes marked decreases in the inorganic P and K of blood serum accompanied by a decreased excretion of P in the urine which is followed by an increased compensatory elimination. They also presented evidence which indicates that the organic P of muscle is increased under the influence of insulin and at the time when the inorganic P of the blood is decreased in amount. These authors also recorded three experiments on normal individuals who responded with decreased amounts of inorganic P of the blood serum after the ingestion of glucose. Strychnine convulsions in rabbits caused a marked increase in the phosphate of the blood and a decrease in the content of the organic P of muscle. Embden and Grafe (9) have shown that there is an increased output of P in the urine as a result of work which presumably causes a breaking down of "lactacidogen."

In this paper we record results which bring additional support to the belief that a certain phase or phases of carbohydrate metabolism are intimately connected with the metabolism of P. The

TABLE I

Composition of Blood and Urine. Glucose Tolerance Experiments.

Date.	Time.	Blood sugar.	Plasma inorganic P.	Urine		Insulin.
				P	Sugar.	
		Subject H. ♀ 50 kg.				
1923–24	*hrs.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per hr.*	*mg. per hr.*	*kg. units*
Oct. 10	Fasting.	84	3.34	20.0	17.4	0
	1	120	2.95	19.0	18.7	
	2	106	3.09	29.3	17.3	
Nov. 2	Fasting.	88	3.16	24.2	15.3	40
	1	48	2.72	14.3	13.7	
	2	40	3.06	3.8	14.1	
Jan. 16	Fasting.	100	3.19	18.4	9.9	0
	1	100	2.98	31.3	13.1	
	2	100	2.65	19.5	14.2	
" 23	Fasting.	100	2.53	16.8	14.6	40
	1	55	2.69	10.4	18.5	
	2	76	1.86	1.3	21.2	
	3	52	2.03	0.7	19.5	
		Subject N. B. ♂ 80 kg.				
Jan. 28	Fasting.	120	2.88	8.0	14.7	0
	1	120	2.74	12.2	21.7	
	2	120	2.69	8.6	19.2	
" 31	Fasting.	110	3.09	10.8	15.6	40
	1	110	2.65	9.1	19.2	
	2	80	2.60	5.6	19.1	
	3	68	1.94	2.1	15.2	
		Subject T. ♀ 65 kg.				
Jan. 29	Fasting.	112	3.55	6.6	13.3	0
	1	214	3.03	11.9	28.1	
	2	112	2.78	13.5	56.8	
Feb. 1	Fasting.	104	3.33	11.1	17.1	40
	1	76	3.63	12.6	21.0	
	2	76	3.08	2.3	20.2	
	3	40	2.54	2.1	21.8	

TABLE I—*Continued.*

Date.	Time.	Blood sugar.	Plasma inorganic P.	Urine.		Insulin.
				P	Sugar.	
Subject J. ♀ 50 kg.						
1923-24	*hrs.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per hr.*	*mg. per hr.*	*kg. units*
Jan. 15	Fasting.	100	2.54	35.2	31.0	0
	1	100	3.40	17.0	17.2	
	2	80	3.51	16.4	20.0	
" 22	Fasting.	100	3.37	19.5	11.5	40
	1	60	3.40	19.5	11.0	
	2	60	2.30	9.0	11.7	
Subject C. ♀ 53 kg.						
Jan. 18	Fasting.	110	3.63	19.2	12.4	0
	1	114	3.67	14.0	12.5	
	2	100	3.63	13.6	11.7	
" 24	Fasting.	100	3.74	12.5	11.8	40
	1	52	3.18	12.5	12.3	
	2	56	2.32	3.9	13.6	
Subject M. ♂ 57 kg.						
Jan. 3	Fasting.	86	2.48	7.7	19.2	0
	1	162	3.14	9.1	23.4	
	2	112	3.30	16.4	33.8	
" 7	Fasting.	100	3.20	14.5	24.0	40
	1	116	2.92	14.7	28.4	
	2	94	3.47	6.9	31.3	
Subject A. B. ♂ 70 kg.						
Jan. 4	Fasting.	110	3.50	24.7	22.8	0
	1	114	3.32	11.3	13.6	
	2	116	4.00	14.6	19.6	
" 8	Fasting.	104	4.17	28.7	28.8	40
	1	76	3.07	35.1	35.9	
	2	72	2.98	34.4	27.1	

TABLE I—*Concluded.*

Date.	Time.	Blood sugar.	Plasma inorganic P.	Urine.		Insulin.
				P	Sugar.	
			Subject M. ♀ 57 kg.			
1923–24	*hrs.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per hr.*	*mg. per hr.*	*kg. units*
Jan. 14	Fasting.	100	3.40	5.8	11.4	0
	1	128	3.09	12.9	14.0	
	2	110	4.32	18.0	16.4	
" 21	Fasting.	100	2.62	10.1	14.1	40
	1	100	2.78	10.2	13.1	
	2	80	2.45	6.9	13.9	
			Subject B. ♀ 66 kg.			
Nov. 14	Fasting.	112	3.55	25.2	13.9	0
	1	154	2.87	24.5	14.4	
	2	92	2.99	25.2	17.6	
" 15	Fasting.	124	4.67	14.0	15.5	40
	1	110	6.83	17.8	15.6	
	2	76	3.91	15.8	16.9	
Dec. 11	Fasting.	100		15.1	12.6	40
	1	92		13.1	13.9	
	2	64		7.9	15.5	
	3	40		4.8	15.6	
" 14	Fasting.	98	3.42	13.0	18.4	40
	1	106	3.71	14.4	16.0	
	2	70	2.61	5.6	22.6	
	3	40	2.14	1.9	14.5	
			Subject S. ♂ 70 kg.			
Jan. 30	Fasting.	116	2.68	14.0	18.1	0
	1	124	2.68	16.5	15.9	
	2	112	2.06	9.7	17.0	
Feb. 5	Fasting.	100	2.62	9.1	14.7	40
	1	64	2.21	8.3	16.1	
	2	32	1.88	2.6	16.1	
	3	32	1.97	1.3	16.7	

findings indicate that a compound of hexose with phosphoric acid, similar to the "lactacidogen" of Embden, is an intermediate product in carbohydrate metabolism.

Plan of Experiments and Methods.

The problem has been attacked by two different means of approach. We have studied the effects of the first Janney and Isaacson (10) glucose tolerance test upon the blood sugar and the inorganic P of the blood plasma and upon the rates of excretion of P and reducing sugar in the urines of normal individuals. The changes produced by the subcutaneous injection of 1 cc. of insulin containing 40 kilo units or 60 clinical units were then determined. The injections of insulin were given just after the initial blood had been drawn and immediately before the glucose was drunk.

We have also studied the excretion of sugar and of P in the urines of three normal individuals eating a standard basal diet. The effects of insulin given in increasing amounts with this diet were observed. The diet eaten had the following composition: protein, 74 gm.; fat, 81 gm.; carbohydrate, 216 gm.; P, 1.61 gm.; and N, 11.8 gm. All the constituents were divided equally between the meals, the hours for which were 8 a.m., 12.30 p.m., and 6 p.m. The urines were collected in 2 hour periods throughout the day from 8 a.m. until 10 p.m. The remainder from 10 p.m. to 8 a.m. served as the night specimen.

The method of Benedict and Osterberg (11) was used for the determination of sugar in the urine, that of Shaffer and Hartmann (12) for blood sugar, and the colorimetric method of Bell and Doisy (13) for the estimation of P.

Glucose Tolerance Experiments.

Without Insulin.—The results of the glucose tolerance experiments are shown in Table I. The excretion of total sugar per hour by nine individuals in the 1st and 2nd hours after glucose ingestion ranged from 11.7 to 33.8 mg., with an average of 17.6 mg. Subject T. showed a sharp rise in blood sugar at 1 hour, from 112 to 214 mg., returning to 112 mg. at 2 hours. Her sugar output was 28.1 mg. in the 1st and 56.8 mg. in the 2nd hour after glucose. These values have not been used in calculating

the average, since the high blood sugar and the increased elimination of sugar indicate a slight defect in the carbohydrate metabolism of this subject. It is interesting to note that the sugar output of this subject after insulin corresponds to that of the others.

The blood sugar values are those usually noted for normal individuals. The inorganic P of the blood plasma and the excretion of P in the urine reveal no definite behavior; there may be an increase, a decrease, or no change.

With Insulin.—During the 1st and 2nd hours after glucose ingestion and the injection of insulin the excretion of sugar ranged from 11.0 to 35.9 with an average of 18.2 mg. per hour. Insulin, therefore, appeared to cause no appreciable change in the rate of sugar elimination after glucose ingestion. In all the urines the amounts of non-fermentable sugar were determined. The values for non-fermentable and fermentable sugar were not changed in any definite manner by insulin and have not been recorded.

The blood sugar values at the end of 1 hour ranged from 48 to 116 mg., at 2 hours from 32 to 94 mg., and at 3 hours from 32 to 68 mg. per 100 cc. The characteristic symptoms accompanying insulin hypoglycemia were not marked in spite of the low blood sugars produced. The most noticeable reaction was observed in the case of Subject N. B. whose blood sugar at the time was 68 mg. per 100 cc. The symptoms noticed by him were rapid pulse, perspiration, and tremor. On the other hand, Subject S. whose blood sugar at 1, 2, and 3 hours was 64, 32, and 32 mg. per 100 cc. experienced no symptoms other than a feeling of weakness. These observations agree with those of clinicians who find that the symptoms of insulin overdosage occur at different levels of blood sugar. All the subjects became ravenously hungry before the completion of the tests, a hunger such as one rarely naturally experiences. The decreases in the inorganic P of the blood plasma after insulin ranged from 6 to 38 per cent in twelve experiments. Quite often there was at first a slight increase in the amount of inorganic P in the plasma followed by the customary drop. Much more striking were the effects upon the excretion of P. Under the conditions present the rate of P excretion fell to 2 mg. and in one case to less than 1 mg. per hour in the 3rd hour following glucose and insulin. Chart 1 portrays graphically the changes which occur in a typical response to insulin.

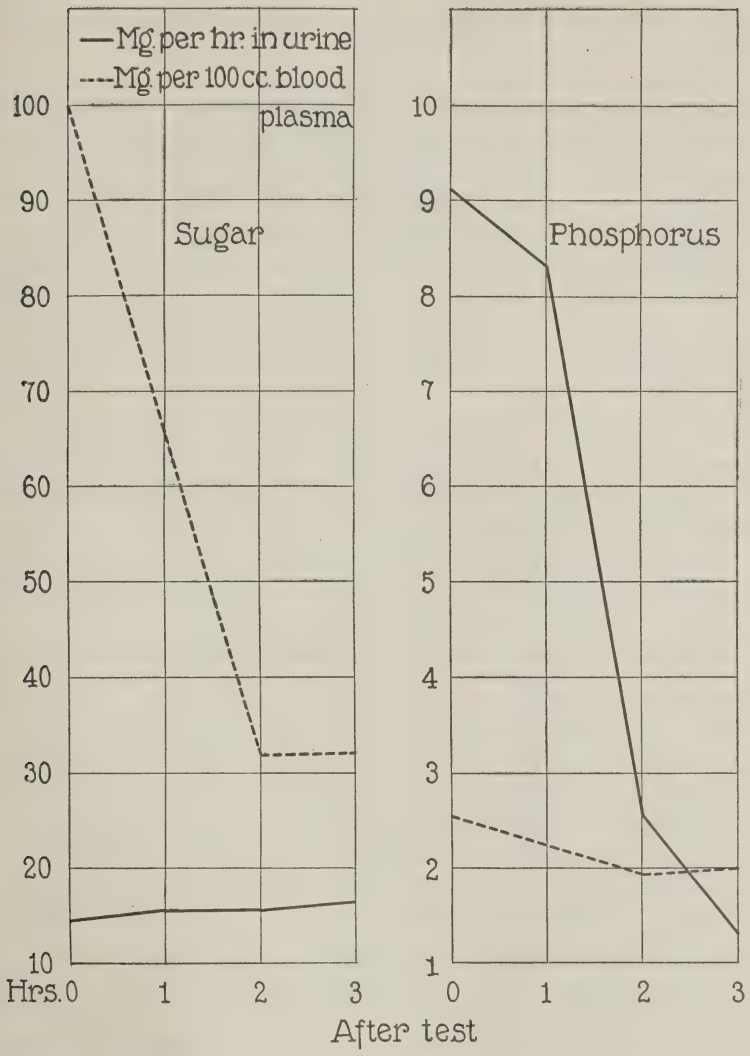


CHART 1. Subject S. Glucose tolerance test with 40 kilo units of insulin. Changes in the sugar and inorganic P of blood and urine resulting from the ingestion of glucose and the injection of insulin.

Effects of Insulin on the Excretion of Sugar and Phosphorus in the Urine.

Chart 2 pictures the hourly rates of excretion of sugar and P in the urines of two subjects living upon the basal diet previously described. The values obtained for several days on this diet have been used in making the curves given in the chart. However, there is but little difference in the shape of the curves, especially that for P, for any of the days on the basal diet. There follow in the chart the changes produced in the curves by superimposing insulin in increasing amounts upon this diet. The insulin was injected in equal doses at 8 a.m. and 6 p.m.

Subject H.

Without Insulin.—The first decided peak in the excretion of sugar came in the 2 to 4 p.m. period followed by a drop in the 4 to 6 p.m. period. The highest rate occurred in the 6 to 8 p.m. period. Glycuresis following meals was not marked.

The P excretion showed a diminishing rate in the forenoon with the first peak in the 12 to 2 p.m. period and a decided peak in the 6 to 8 p.m. period (the highest rate of the day).

With Insulin.—Insulin did not materially alter the shape of the sugar curves.

The first marked effect of insulin was to cause a greater excretion of P for 2 days. The increase of the 1st day was due to an augmented rate beginning in the 10 a.m. to 12 n. period and lasting throughout the night with exception of the 6 to 8 p.m. period which was lower than the corresponding basal period. The 2nd day showed an increased rate during the forenoon and during practically the entire day excepting that the 8 to 10 p.m. and night rates fell below the corresponding basal periods. The 24 hour excretion of P was significantly decreased in the 3 remaining days. This happened because of decreased rates in all but the 2 to 4 and 4 to 6 p.m. periods. The striking points are that the forenoon rate was markedly decreased; the peak in the 6 to 8 p.m. period was eliminated and the rate for the 8 to 10 p.m. period became less than that for the night period which was also decreased from the basal average value. The lowered values in the 10 a.m. to 12 n. and 8 to 10 p.m. periods coincide with the same time interval after insulin (from 2 to 4 hours after the injection).

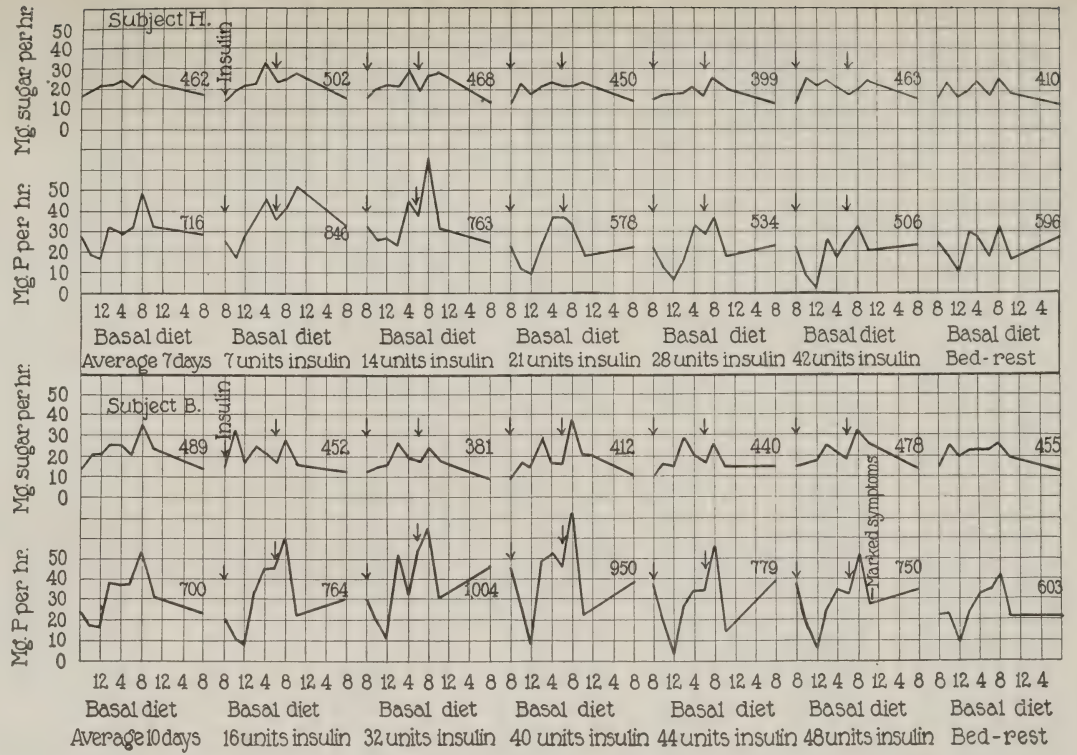


CHART 2. Curves of excretion of sugar and P in the urine. Showing the effects of insulin and of muscular activity.

Subject B.

Without Insulin.—The curve of sugar excretion showed the first rise in the 8 to 10 a.m. period, another in the 12 n. to 2 p.m. period, a drop to the forenoon level in the 4 to 6 p.m. period and a sharp peak in the 6 to 8 p.m. period. This subject shows plainly the glycuresis following meals which was first emphasized by Benedict.

The P excretion showed a decreasing rate during the forenoon, reaching the lowest point of the 24 hours in the 10 a.m. to 12 n. period. There was a sharp rise following the noon meal in the 12 n. to 2 p.m. period from which time the rate remained practically constant until the 6 to 8 p.m. period when there was a marked increase (the highest rate of the 24 hour period).

With Insulin.—Insulin did not affect the shape of the sugar curves.

The effect of insulin on the P was to cause an increased excretion for the 24 hour periods. There was a slight increase the 1st day, a marked increase the 2nd day, followed by a gradual decrease for the remaining 3 days. In every case there was a marked decrease in the forenoon rate especially in the 10 a.m. to 12 n. period. The remainder of the day with the exception of the 8 to 10 p.m. period tended to be higher than the basal. There was a sharp increase in the night rate which in the last 2 days kept the 24 hour amount above that for the basal diet. The rate for the 8 to 10 p.m. period was the same 1 day and less in the remaining 4 days than for the basal diet. The decreased values in the 10 a.m. to 12 n. and in the 8 to 10 p.m. periods coincide with the same time interval after insulin (from 2 to 4 hours after the injection).

Subject S.

Determinations of total N and uric acid besides those of sugar and P were made on the urines of this subject. We were interested to learn whether the preliminary increase in P excretion caused by insulin was associated with an increased protein or nuclear metabolism. These values are presented in Chart 3.

Without Insulin.—The rate of sugar excretion during the day periods was very constant, varying only from 20.2 to 26.0 mg. per hour. The night rate was 14.8 mg. per hour.

There was a very slow rate of excretion of P in the forenoon reaching the low value of 3.8 mg. per hour in the 8 to 10 a.m.

period. The rate then increased to the first peak in the 12 n. to 2 p.m. period, followed by a slight drop in the 2 to 4 p.m. period. The rate then returned to the former level where it remained during the rest of the day and the night. This curve was entirely unlike that of the other two subjects who showed decided peaks in the 6 to 8 p.m. period and marked decreases in the night rate.

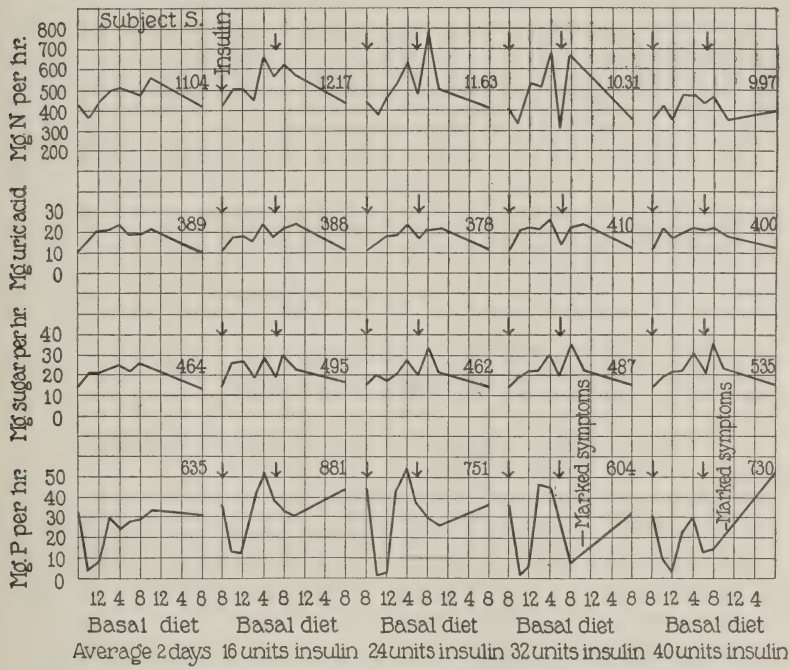


CHART 3. Curves of excretion of N, uric acid, sugar, and P in the urine. Showing the effects produced by insulin.

The lowest rate of N excretion was in the 8 to 10 a.m. period. It then increased reaching the first high point in the 2 to 4 p.m. period after which there was a slight decrease until the 8 to 10 p.m. period when the highest point of the 24 hours was reached. The N elimination was the highest about 3 to 4 hours after the meals.

The shape of the uric acid curve was similar to that of N. The highest rate of uric acid excretion was reached in the 2 to 4 p.m. period instead of in the 8 to 10 p.m. period as in the case of N.

With Insulin.—Insulin caused an increased elimination of P for the first 2 days. On the 3rd day the value fell below the basal only to rise above it on the next and final day. The increased excretion of the first 2 days was due to an augmented rate in the 2 to 4 p.m. period and in the night period. The increased elimination of the last day was caused by a greatly increased night rate (about 60 per cent more than the basal night rate). The rate during the day on the last day was markedly decreased. The rate in the 6 to 8 p.m. period on the 3rd insulin day reached a very low level. The peak of the P curve comes in the 2 to 4 p.m. period (from 6 to 8 hours after the injection of insulin).

The N intake of this subject was 11.9 gm. daily. The excretion of N for the 2 days on the basal diet averaged 11.04 gm. The addition of 16 units of insulin resulted in an output of 12.17 gm. (a negative balance). After the 1st insulin day and with increasing amounts of insulin there was a progressive decrease in the excretion of N until 9.97 gm. were eliminated on the last day.

Insulin does not appear to have influenced nuclear metabolism as judged by the values obtained for uric acid.

Effects of Insulin on the Different Forms of Urine Sugar.

Subject H.—There was very little change in the amount of total sugar excreted when insulin was given. 8.1 per cent less fermentable sugar and 2.8 per cent more non-fermentable sugar were excreted during the insulin period. The percentage of fermentable sugar decreased from 37.2 on the basal diet to 34.6 on the basal diet with insulin.

Subject B.—All forms of sugar were excreted in less amount when insulin was given. There was 11.5 per cent less of total sugar, 19 per cent less of fermentable, and 6.7 per cent less of non-fermentable sugar. The percentage of fermentable sugar decreased from 38.7 on the basal diet to 35.3 on the same diet with insulin.

Subject S.—This subject responded with an increase in all forms of sugar when insulin was administered. The increases over the basal diet were the following: total sugar, 6.7 per cent; fermentable sugar, 13.6 per cent; and non-fermentable sugar, 0.8 per cent. The percentage of fermentable sugar on the basal diet was 45.9 and with insulin this increased to 48.9 per cent of the total.

Influence of Muscular Activity on the Excretion of Phosphorus.

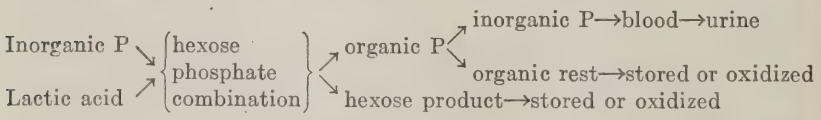
Embden and Grafe (9) have reported that muscular activity causes an increased excretion of P in the urine. In Chart 2 are recorded the results of our experiments in this connection. It is seen that both of these subjects excreted less P when at bed-rest than they did under the conditions of ordinary muscular activity.

DISCUSSION.

The experiments with glucose ingestion and the injection of insulin show very clearly a decrease in the inorganic P of the blood plasma accompanied by a diminished rate of excretion of P in the urine. These changes occur at the time of hypoglycemia when one may expect carbohydrate metabolism to be proceeding at a rapid rate. The decrease in the inorganic P of the plasma was sometimes not manifested until the 2nd hour after the insulin and glucose were administered. This constituent was occasionally increased in amount at the end of the 1st hour. The case of Subject B. is interesting in this connection. She showed a marked increase in the phosphate of the plasma 1 hour after the administration of glucose and insulin on November 15. The rate of P excretion in the urine was also slightly increased. This subject had taken the regular glucose tolerance test the preceding day and had also eaten doughnuts immediately afterward, a hearty lunch, and dinner and cake in the evening. The carbohydrate stores of this subject must have been satiated. This test was repeated at a later date under standard conditions with typical changes recorded for the phosphate of the plasma and the urine. These experiments indicate that insulin may under certain conditions cause an increase in blood phosphate but that this phase is usually missed or nullified by the reaction proceeding at a greater rate in the other direction. One may assume that when the stores of carbohydrate are filled the first action of insulin is to cause a breaking up of the hypothetical hexose phosphate combination present in the tissues. Our findings with the regular glucose tolerance test do not agree with those of Harrop and Benedict (8) who observed that the inorganic P of the serum was decreased. Our results in this connection showed no definite change, there sometimes was an increase, a decrease, or no change. No explanation is apparent for this discrepancy.

The effects produced by adding insulin in increasing amounts to a uniform basal diet are very instructive. The salient points follow. Insulin causes a greatly increased excretion of P for 1 or 2 days. This is accompanied by an augmented elimination of N. The excretion of P and N then gradually decreases until the values may become less than for the standard diet. The morning injection of insulin effects a greatly lessened rate of excretion of P during the forenoon. The shape of the P curve is in all cases greatly altered. Insulin tends to cause a decreased excretion of P during the day and an increased elimination during the night. It may be assumed that these effects are produced by inorganic P being withdrawn from the blood to form the hexose phosphate combination in the other tissues and when the effects of the insulin have been spent this compound breaks up thus releasing phosphate into the blood and thence into the urine.

The reactions presented in the introduction of this paper postulate that the phosphate-carbohydrate combination is an intermediate between lactic acid and glycogen. It is perhaps well to emphasize that Embden does not consider "lactacidogen" to be such an intermediary product between glycogen and lactic acid. Embden and his school believe that the glycogen of muscle is to be looked upon as a carbohydrate reserve stored for times of insufficient supply. They have presented evidence which indicates that the source of the "lactacidogen" P is the organic rest P of muscle. This interpretation leaves open the question of the source of the carbohydrate moiety of "lactacidogen." For a discussion of this phase of the question the reader is referred to the papers by Adler (14, 15). It seems to us that the scheme presented below better explains the known facts dealing with the changes produced by insulin than does the assumption that the hypothetical combination is intermediate between lactic acid and glycogen. Neither does this plan necessarily omit glycogen from being one of the substances involved. The outline which follows may represent the changes occurring under the influence of insulin.



The decrease of inorganic P in the blood resulting from the action of insulin is explainable by assuming that the hexose phosphate combination is formed in the tissues other than the blood. When this combination disintegrates then inorganic P should reappear in greater amounts in the blood and increased quantities of P should be excreted in the urine. These facts have all been demonstrated excepting the increased phosphate in the blood which certainly must occur. This reaction may be considered reversible. The changes produced by strychnine would be represented by the reaction proceeding toward the left.

In the past many attempts have been made to determine a parallelism between the rates of excretion of N and P in the urine. It is now easy to understand why this was impossible of accomplishment. The rate of excretion is undoubtedly chiefly influenced by the carbohydrate metabolism and not by the metabolism of protein or of nuclear material.

Our experiments show that when insulin was first given there was an increased excretion of N. Continued administration of insulin caused a gradual decrease in the elimination of N to a point at which storage must have been taking place. The preliminary greater excretion of N reminds one of the stimulating effect of thyroxin upon protein metabolism. However, since this was not a sustained action one is forced to consider other possibilities. The cause for the gradually decreasing excretion of N with the continued administration of insulin may have been a protein-sparing action of the considerable amounts of lactic acid formed. Kocher (16) has shown that lactic acid is an efficient sparer of protein. The preliminary increased excretion might also be referred to the release of reserve nitrogenous material. The fact that greater amounts of P also accompany this augmented elimination of N suggests that there is a greater metabolism of either protein or nuclear material or both. The values recorded for uric acid seem to eliminate nuclear metabolism from this consideration. Our findings do not permit an answer to this question.

The excretion of normal urine sugar does not appear to be materially changed by the injection of insulin. This applies to the amount of total sugar and to its distribution. Two of the subjects excreted less and one subject more fermentable sugar when insulin was administered. These results indicate that the amount

of normal urine sugar is quite independent of the blood sugar content when this is below the renal threshold. The blood sugar of these subjects was below the fasting level of 100 mg. per 100 cc. many times during the day. This fact is known because of the symptoms of insulin overdosage which occurred and is further substantiated by determinations of the blood sugar concentration.

SUMMARY.

Insulin administered to normal individuals before glucose ingestion causes a marked decrease in the inorganic P of blood plasma. The rate of excretion of P in the urine is greatly lessened at the same time. These changes occur during the period of hypoglycemia.

When insulin is superimposed upon a standard diet the rate of excretion of P in the urine is altered to a large extent. These changes in rate are referable to the injection of the hormone. Insulin at first causes an increased elimination of N and P, but with continued administration these values tend to become less than for the standard basal diet.

The relation of these findings to carbohydrate metabolism is discussed. They bring additional support to the hypothesis that a combination of hexose with phosphoric acid is an intermediate product in the metabolism of carbohydrate.

The excretion of normal urine sugar is not appreciably affected by the injection of insulin. The amount of normal urine sugar seems to be quite independent of the blood sugar concentration when this is below the renal threshold.

Muscular activity causes a greater excretion of phosphorus in the urine.

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ON THE MECHANISM OF THE CATALYTIC ACTION OF IRON SALTS. I.

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The idea that iron salts act as catalyzers in biochemical processes and play an important part in respiration, particularly, has gained in significance lately through the work of various investigators.

The mechanism of this action of iron remained obscure, however, and one was content with the simple assumption that the variable oxidation stages

$$\overset{\text{II}}{\text{Fe}} \rightleftharpoons \overset{\text{III}}{\text{Fe}}$$

were responsible for the specific catalytic power of the iron salts.

This general assumption seems, however, to be of very doubtful validity when one considers that on a change from the ferrous to the ferric state, a very stable form is assumed which can be brought back to the ferrous state only with the use of very considerable amounts of energy.

The reduction of trivalent to divalent iron is very difficult to bring about in the ordinary ionic salts of iron. Iron bound in a complex ion, on the other hand, is in general easily brought into each of the oxidation stages, as Baudisch[1] has previously shown. Visible and ultra-violet radiation or hydrogen peroxide possesses the power to reduce the trivalent iron of certain complex compounds into the divalent form. The divalent iron has quite distinct properties from the trivalent. For example, it is able to absorb oxygen from the air and link it coordinatively. The auxiliary valence forces are usually stronger than in the trivalent form and the iron nucleus is, therefore, able to draw the various inorganic

[1] Baudisch, O., *Biochem. Z.*, 1918, xcii, 189.

and organic radicals or compounds into the inner sphere selectively. As a result, we can get a very large number of more or less stable complex salts with divalent iron in the nucleus.

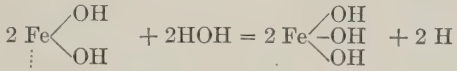
Ferrous hydroxide, obtained by precipitation of 1 mol of ferrous sulfate with 2 mols of sodium hydroxide, is extraordinarily autoxidizable and it is almost impossible to obtain it in snow-white form free from oxygen. Manchot and Herzog,[2] after a long and thorough study of the autoxidation of iron compounds, come to the conclusion that, unlike all the other metals investigated, no hydrogen peroxide is formed as an intermediate or end-product of these autoxidation processes.

For the explanation of the mechanism of the autoxidation process in ferrous salts it is naturally of importance to determine whether or not hydrogen peroxide is formed. Manchot[3] tried to show that active oxygen is formed which can oxidize other substances present in the solution. He used the readily oxidizable arsenic acid in very great excess in order to capture the oxygen activated by the ferrous iron. He assumed that the iron particles at the moment when the oxygen, either "activated" or given up by the superoxide initially formed, are surrounded by very many acceptor particles and, therefore, collide much more frequently with the acceptor than with other ferrous particles. This consideration led to the desired goal. If in an oxygen atmosphere one mixes iron sulfate solution with potassium hydroxide in which an excess of arsenic oxide is dissolved and shakes the mixture, the red-brown color of the ferric hydroxide first appears when twice as much oxygen has been absorbed as corresponds to the change from the ferrous to the ferric stage.

In this way Manchot showed that arsenic oxide present in excess could be oxidized by ferrous salt plus air or oxygen. He also showed that in the oxidation of ferrous compounds the proportion of the activated oxygen to that involved in the formation of iron oxide was 1:1. It was, therefore, very probable, as Manchot thought, that the peroxide FeO_2 was formed. According to

[2] Manchot, W., and Herzog, J., *Z. anorg. Chem.*, 1901, xxvii, 404.
[3] Manchot, W., *Z. anorg. Chem.*, 1901, xxvii, 420.

Engler's theory the iron atom acts as a pseudo-autoxidator and the course of the reaction is as follows:

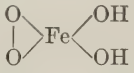


$$2\,H + O_2 = H_2O_2$$

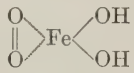
Just[4] has made some very important and interesting investigations on the autoxidation of ferrous salts. He arrived at results which are in conflict with the hypothesis of Manchot and the observations of Engler. Just studied the autoxidation of an aqueous solution of ferrous bicarbonate from the standpoint of the kinetics of the reaction. He determined the concentrations of the substances involved. It was found that an entire oxygen molecule takes part in the first stage of the oxidation. It was not possible to determine in which way the molecular oxygen combines with the ferrous salt, but it was shown that, in this reaction, 1 and not 2 molecules of the ferrous salt reacts with the molecular oxygen. As a result of the hydrolysis of the ferrous bicarbonate

$$Fe(HCO_3)_2 = Fe(OH)_2 + 2\,CO_2$$

the hydrolytic compound Fe $(OH)_2$ takes up 1 oxygen molecule forming an intermediate compound. According to Engler and Weissberg[5] this intermediate product is a "moloxyd"



According to Bredig, it is a peroxide of the form



This peroxide, in contrast to Manchot's assumption, is a quadrivalent iron derivative. The kinetic method leads us to postulate, not the intermediate formation of FeO_2 or Fe_2O_5, but rather the existence of the peroxide FeO_3.

[4] Just, G., *Ber. chem. Ges.*, 1907, xl, 3695; *Z. physik. Chem.*, 1907, lxiii, 385.

[5] Engler, C., and Weissberg, J., Kritische Studien über die Vorgänge der Autoxydation, Brunswick, 1904.

As to the further course of the reaction, Chandra has made important contributions. He showed that ferrous carbonate is soluble in solutions of sodium, potassium, or rubidium bicarbonate as well as ammonium carbonate. It was also shown that these solutions, standing in air, are spontaneously oxidized to complexes having ferro-ferric ions of the form

$$\left\{ {}^{II}Fe_2 \cdots\cdots {}^{III}Fe \cdots\cdots (CO_3)_3,\ O,\ H_2O \right\}.$$

The hitherto unknown alkali salts of these ions were isolated and examined in more detail. By dissolving these ferro-ferric carbonates of the type Fe_6O_7 with acids, the sulfates and the chlorides, unknown up to that time, of the base of $Fe_6O_7 \cdot XH_2O$ were found. It was also shown that in a solution of the hydrate $Fe_3O_4 \cdot XH_2O$, a complex base of this composition may be assumed to exist.

All observations on the autoxidation of ferrous salts, therefore, indicate that the oxidation of the ferrous ion to the ferric ion by air or oxygen is a complicated reaction extending through several stages which have not been sufficiently investigated in either their chemical or physical aspects. But it is, particularly from a biochemical standpoint, important to obtain further information as to the mechanism of this oxidation. It is known with considerable certainty that the catalytic action of the iron in respiration and many other biologically important processes is especially related to the properties of the ferrous atom or ferrous ion and not so much to the ferric atom or ferric ion.

Up to this time, nothing was known about the mechanism of the oxidation of inorganic or organic compounds with ferrous salts and air or oxygen. Such an oxidation could not be placed in direct analogy with the oxidation of inorganic and organic compounds by hydrogen peroxide; for the active oxygen is never present in excess and the substance to be oxidized is, so to speak, embedded in the strongly reducing ferrous compound.

An oxidation-reduction system of the type considered is represented by the mixture of ferrous sulfate and sodium bicarbonate first used by Baudisch. If, for example, sodium lactate with ferrous bicarbonate in an excess of sodium bicarbonate is shaken with air, there appears an odor of acetaldehyde at the very

beginning of the entrance of air and the coloring of the white ferrous bicarbonate. After a few minutes shaking the presence of pyruvic acid may be detected. This experiment alone brings out the extraordinary fact that in spite of the great excess of molecules and ions of ferrous bicarbonate or ferrous hydroxide, 2 hydrogen atoms of lactic acid are removed by oxidation and pyruvic acid is formed. The reaction is similar in its course to that carried out by Wieland[6] on the dehydrogenation of lactic acid with palladium black. The principal product is pyruvic acid, together with small amounts of acetaldehyde and acetic acid. The splitting of the hydrogen is explained by Wieland as due to the primary activation by the finely divided palladium. In this case no oxygen is necessary, while in the dehydrogenation of lactic acid with ferrous bicarbonate or ferrous hydroxide the necessity of its presence is absolute. As yet no explanation exists as to the manner in which the dehydrogenation or oxidation of organic compounds by ferrous salts takes place.

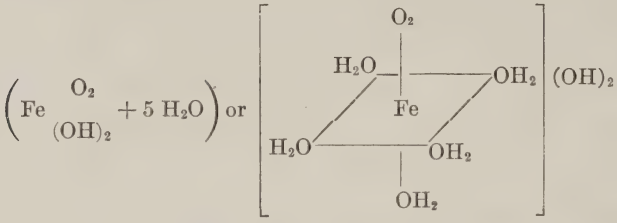
In the processes of oxidation or dehydrogenation with ferrous salts and air it is very probable that the molecular oxygen loosely bound to the iron may act as a dehydrogenation agent towards the oxidizable substance present in the mixture. Or it may be that the iron nucleus of the precipitated iron hydroxide molecule or the iron in solution is responsible for the dehydrogenation. In the latter case, the iron would act in the same way as the finely divided palladium black.

It is necessary, first of all, to decide between these two alternatives. It amounts to deciding this question: Is the oxidation process to be referred to the activation of oxygen or to the activation of the released hydrogen atoms in the sense that Wieland[6] explains the action of palladium black?

Our experiments to date have made it clear that both factors play a part, for it is only the oxygen, which has been "activated" by iron, that has the property of adding to itself organic and inorganic groups or compounds. Ordinary molecular oxygen cannot do so. Then, too, the only organic compounds which are

[6] Wieland, H., *Ber. chem. Ges.*, 1913, xlvi, 3332.

dehydrogenated under the given conditions are those special ones whose hydrogen atoms show an affinity to the iron peroxide

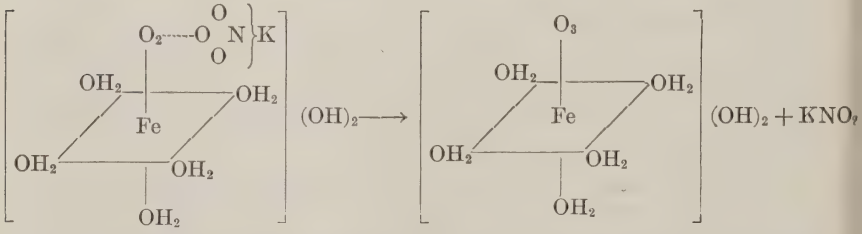


Coordination formula.

initially formed.

We shall now describe the experiments by which it has been shown that, initially, a molecule of oxygen is added to 1 molecule of ferrous salt. The oxygen molecule, thus activated, is able to link certain compounds in a coordinative manner and make them more active; in other words, *the oxygen molecule attached to the iron nucleus forms a new center of forces.*

The reduction of nitrates with molecular oxygen in the presence of iron salts as first described by Baudisch[7] proved to be of great value in this study. For in this chemical process it can be assumed that the nitrate molecule is primarily linked to the activated oxygen molecule, whereupon the chemical changes take place as indicated in the scheme:

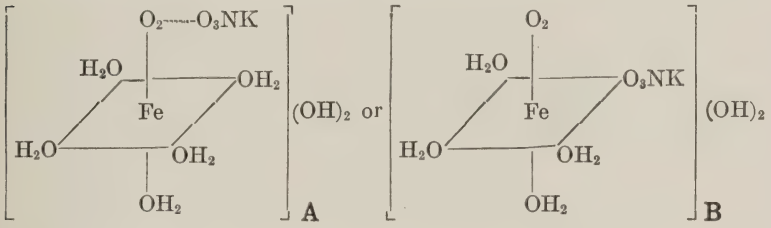


That the mechanism indicated in this scheme actually takes place will be seen from our following experimental results.

At ordinary temperatures, alkali nitrates are not affected by nascent ferrous hydroxide. It is the oxygen dissolved in the

[7] Baudisch, O., and Mayer, P., *Biochem. Z.*, 1920, cvii, 1. Mayer P., Inaugural dissertation, Technischen Hochschule, Zurich, 1920.

water which reduces the nitrate to nitrite.[8] The quantity of reduced nitrite is proportional to the amount of dissolved oxygen. Or, what amounts to the same thing, the reduction is proportional to the partial pressure of the oxygen. The experiment, therefore, proves that oxygen is necessary to the reduction of nitrates, but it does not make the mechanism any clearer. The question remains: Is the alkali nitrate drawn into the field of force of the molecular oxygen (A) or is it drawn into the field of force of the iron nucleus (B)? For, in the latter case, it is perfectly possible that the oxygen linked with the iron disturbs the forces and permits the entrance and linkage of the nitrate molecule.



In the case of alkali nitrites it was previously shown by Baudisch[9] that it is the nitrogen of the nitrite molecule which is directly linked to the iron nucleus and that molecular oxygen is not necessary for the reduction. In the case of the nitrates it seemed to be impossible to determine the mechanism by further chemical experiments and considerations or by physical measurements such as the conductivity, light absorption, etc.; since it is only the *freshly precipitated* colloidal ferrous hydroxide-peroxide which brings about the reduction of the nitrate in neutral or weakly alkaline solution and not the isolated compound itself.

A consideration of the magnetic properties of iron compounds was resorted to in order to get further light on the matter. It is known[10] that the practically non-magnetic, white ferrous hydroxide becomes black in color and strongly ferromagnetic on absorption of oxygen, but on further absorption of oxygen it turns into the

[8] Baudisch, O., *Ber chem. Ges.*, 1921, liv, 406.

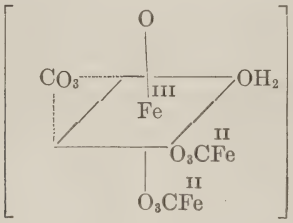
[9] Baudisch, O., *Ber. chem. Ges.*, 1918, li, 793.

[10] Hilpert, S., *Ber. chem. Ges.*, 1909, xlii, 2248. Quartaroli, A., *Chem. Zentr.*, 1917, i, 729.

red and weakly magnetic ferric hydroxide. This fact shows that molecular oxygen acting on ferrous hydroxide forms intermediate *polynuclear* complex compounds which are again broken up on further oxidation. Similarly, if sodium bicarbonates are used instead of alkali hydroxides, iron goes in solution with the formation of polynuclear magnetic compounds of the definite constitution

$$\left\{ {}^{II}Fe_2 \cdots\cdots {}^{III}Fe \cdots\cdots (CO_3)O,\ H_2O \right\} Na$$

For the coordination formula we assume the following scheme.



Chandra's compound.[11]

In any case, the experiments make it plain that the *molecular* oxygen dissolved in water stands in direct relation to the formation of polynuclear complex salts and their magnetic properties just as it stands in direct relation to the reduction of nitrates to nitrites.[12]

On the basis of these facts we may assume that if any chemical or physical changes take place in the loosely bound molecular oxygen, a striking change in the magnetic behavior of the newly formed compound should also occur. For example, if the nitrate molecule becomes primarily linked to the iron peroxide oxygen as indicated in the scheme

$$\begin{matrix} O_2 \cdots\cdots O_3NK \\ Fe \\ (OH)_2 \end{matrix} \qquad (I)$$

we cannot expect that at any stage of the reaction we shall have the compound $Fe_3O_4 \cdot XH_2O$ such as is ordinarily formed when air

[11] Chandra, H., Inaugural dissertation, Friederich Wilhelm University, Berlin, 1913.

[12] Baudisch, O., *Ber. chem. Ges.*, 1921, liv, 410.

reacts with ferrous hydroxide. The reason is that the molecular oxygen is, one might say, blocked by the nitrate molecule. The attached nitrate molecule, however, is quickly decomposed, yielding nitrite and an oxygen atom. The latter attaches itself to the molecular oxygen and forms an intermediate compound of the following formula

$$\begin{matrix} & O_2 \cdots\cdots O \cdots\cdots \\ Fe & \\ & (OH)_2 \end{matrix} \qquad \text{(II)}$$

in which the configuration of the 3 oxygen atoms may be similar to that which is known of ozone. We may assume that the oxygen in this form, O_3, is the center of a stronger field of force than if it were oxygen of the ordinary molecular form, O_2. Instead, then, of only 2 unchanged ferrous hydroxide molecules being drawn into the field of force to form $Fe_3O_4 \cdot XH_2O$ we shall have more than 2 such molecules attracted and a *different* magnetic ferro-ferric compound should appear as a result.

In every way this assumption has now been borne out by our experiments.

If one precipitates ferrous hydroxide (1 mol of $FeSO_4$ + 2 mols of NaOH) in the presence of alkali nitrate and air or oxygen, one obtains besides $Fe_3O_4 \cdot XH_2O$ a new, strongly magnetic iron oxide which, as far as our present data show, has the composition

$$3Fe_2O_3 \cdot 2FeO \cdot XH_2O$$

Its chemical and physical properties differ from those of $Fe_3O_4 \cdot XH_2O$. It is more stable to air and it is more resistant to the influence of dilute acids. It is much less soluble in 50 per cent acetic acid. This fact is made use of in separating it from the $Fe_3O_4 \cdot XH_2O$ which is formed, to some extent, in the mixture from which the new oxide is precipitated.

Another striking difference appears in the way they absorb water. At room temperature the $Fe_3O_4 \cdot XH_2O$ can absorb as much as 33 per cent of water while the new oxide absorbs not more than 3 per cent. We are studying this oxide in more detail and the results will appear elsewhere. We are also looking into the question as to how it compares with the magnetic ferro-ferric oxide first described by Haber and Kaufmann.[13]

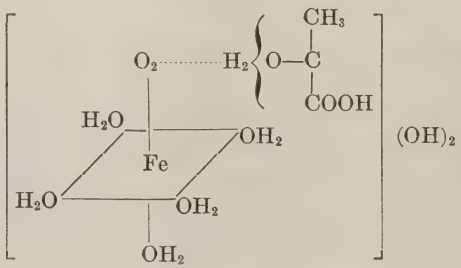
[13] Haber, F., and Kaufmann, A., *Z. Electrochem.*, 1900–01, vii, 733.

In this paper, however, we are especially concerned with the important fact that while forming a new magnetic oxide under the conditions which have been described, we have cleared up the mechanism of the reaction by which nitrates are reduced to nitrites by oxygen and ferrous salts.

It is only on the assumption that the nitrate molecule adds itself to the iron peroxide oxygen according to Scheme I that we can explain the formation of this new, strongly magnetic oxide. For we have convinced ourselves by direct experiment that reagents which oxidize the iron nucleus directly, such as hydrogen peroxide, potassium persulfate, and potassium nitrite, form substances which are not, in the slightest degree, ferromagnetic. We may add that the strong magnetic qualities of the ferro-ferric compounds of iron are determined by the special spatial arrangement of the ferrous and ferric oxide molecules assumed at the instant of its formation. When once formed it retains its ferromagnetic qualities even if the ferrous parts of the compound are subsequently oxidized to the ferric form.

In the remaining discussion other experiments are described which confirm and extend the conclusions reached and throw still more light on the catalytic action of iron salts in oxidation processes.

It has been mentioned that it is possible to change an alkaline solution of lactic acid into pyruvic acid by means of ferrous hydroxide and air. While the mechanism of this biologically important reaction has remained obscure, the observations which we have made make it reasonable to assume that it proceeds in this way: While in its nascent state the peroxide formed by ferrous hydroxide and air links a molecule of lactic acid, as indicated in the following scheme,



and splits off the activated hydrogen atoms with the formation of water and pyruvic acid. To prove that this mechanism actually takes place *magnetism* was again used as a reagent and indicator. When ferrous hydroxide is precipitated in excess of potassium lactate and in the presence of potassium nitrate only a very small portion of the nitrate is reduced to nitrite. Some part of the lactic acid, however, is oxidized to pyruvic acid. A magnetic oxide is not formed in this case, because it is the lactic acid molecule and not the oxygen of the nitrate which has occupied and satisfied the auxiliary valence of the molecular oxygen bound to the iron. If, however, potassium oxalate is used under the same conditions, it is found to be of practically no influence and does not interfere with the reduction of the nitrate by oxygen dissolved in the water. Nor does aniline interfere with the nitrate reduction and the formation of the strongly magnetic oxide. This oxide can be distinguished and separated from the magnetite $Fe_3O_4 \cdot X\ H_2O$ by the fact that it is not soluble or at least difficult to dissolve in a 50 per cent solution of acetic acid.

But if glycerol or grape sugar is substituted for the oxalic acid, a dark precipitate, which is readily dissolved by acetic acid of 50 per cent strength, occurs. Incidentally, it may be mentioned that with this reaction it is easy to differentiate between those compounds which show affinity to the secondary valences of the molecular oxygen linked with the iron nucleus and those compounds which do not.

The new and extraordinarily magnetic compound is formed by nitrates and nascent ferrous hydroxide in, and only in, the absence of those compounds having an equal or greater affinity for molecular oxygen linked to the iron nucleus. The resulting black precipitate, when filtered and washed with 50 per cent acetic acid, remains practically insoluble and is strongly magnetic. On the other hand, if the compound present has a greater affinity for nascent ferrous hydroxide than has "nitrate oxygen"[14] the precipitate formed is readily soluble in the acetic acid.

We are thus face to face with the new fact that ferrous salts, together with air or oxygen, acquire by the coordinative linkage of oxygen, not only the well known reducing power but also the

[14] Baudisch, O., *Ber. chem. Ges.*, 1912, xlv, 2897; 1916, xlix, 1176.

property of acting as oxidizing or better dehydrogenating agents. But its rôle and importance in many other biological processes is still vague and not at all cleared up. It is to be expected that there would be a great variety of chemical processes induced by ferrous salts. The fact, established by Baudisch,[14] that both the ionic and complex salts of ferrous iron behave in different ways towards nitrates and nitrites according to the absence or presence of oxygen is an indication of these variabilities.

Another interesting fact is the correlation between the oxidation of iron salts and the action of carbon dioxide present in the mixture.

Bunte and Schmidt were the first to recognize that it was the concentration of each of the components of ferrous salts, carbon dioxide, and oxygen which determined the rate of oxidation. According to these experiments it also becomes probable that the reaction is of the first order as far as the iron is concerned. This interpretation is in agreement with the observations of Just that have been mentioned.

In nature, it is likely that the presence of carbon dioxide brings about conditions resembling those of an oxygen-free atmosphere. The carbon dioxide protects the ferrous compound from oxidation and it is not until *nascent* ferrous hydroxide has been formed from ferrous bicarbonate by hydrolysis that oxygen can be absorbed and the reactions, as previously described, can begin. In this fact lies the key to an understanding of the observation that a mixture of ferrous sulfate and sodium bicarbonate, that is, ferrous bicarbonate in *status nascendi*, reacts by shaking with air in a special way on certain organic substances present in the reaction mixture.

The changes in the pyrimidines and the formation of indigo from indole by treating indole with ferrous bicarbonate and air have been studied by Baudisch[15] and his coworkers. In the case of cytosine, for instance, water molecules activated by ferrous hydroxide-peroxide do not only hydrolyze the amino group of cytosine with the liberation of ammonia but there also follows an addition of 1 water molecule to the double carbon linkage with the intermediate formation of dihydrobarbituric acid. All these

[15] Baudisch, O., *J. Biol. Chem.*, 1924, lx, 155.

experiments constitute further evidence for the varied character of the action of ferrous salts in the presence of air or oxygen.

Further, it is of particular interest from a physiological standpoint that ferrous salts also have the property of drawing selectively into the inner sphere of the iron nucleus, those compounds which are of an ethylene character or which contain nitrogen. Very many of the substances occurring in nature, such as uracil, cytosine, indole, piperidine, etc., are indeed predestined to form such complex compounds. With the entrance of from one to six groups or whole molecules into the inner sphere of the ferrous nucleus, the possibility is opened up for numberless new reactions. This is because the remaining unoccupied coordination forces are changed both qualitatively and quantitatively.

The selective linking effects of the groups originally bound are also changed. The observation is new and of especial biological interest that the molecular oxygen which is linked in the inner sphere of a ferrous compound is able to bind coordinatively not only unchanged ferrous hydroxide molecules but also a large variety of other substances.

Our knowledge of the new ferromagnetic ferro-ferric oxide formed by the transfer of atomic oxygen from the nitrate to molecular oxygen linked with divalent iron and the additional knowledge of the intermediate compound

$$\left(\begin{matrix} O_3 \\ Fe \\ (OH_2)_5 \end{matrix}\right)(OH)_2$$

is doubtless of far reaching importance in the problem of determining the nature of the coordinative forces in general and, particularly, those in ferrous salts. It is already clear from our experiments on ferromagnetic compounds that a small change in the fields of force about the iron nucleus of the complex, such as the substitution of O_3 for O_2, brings about a very large difference in the magnetic behavior of the compound.

One is forced to conclude that intimate relations exist between the coordination forces and magnetic forces if they are not indeed one and the same thing.

It is hardly mere chance that it is the ferromagnetic metals such as Fe, Ni, Co, Pt, etc., which form such a large number of

complex compounds and that it is the paramagnetic gases O_2 and NO which show particular affinity for these same metals, the affinity of oxygen to iron being a common and striking case.

The idea that magnetic forces associated with the electron orbits play a part in the development of molecules out of two or more atoms is not a new one. It has been developed in considerable detail by Lewis.[16] It leads directly to the notion of electron pairs which is emphasized so much in certain atomic theories. It is supported by the fact that the complex ion retains its identity in solutions and does not suffer the breaking up which is connected with electrostatic coupling of the component parts. Still another indication is the marked influence of addition to and substitution within the complex on light absorption. Light absorption is not affected by electrolytic dissociation. It is easy to see in a general way that the normal states of the electron orbits of, say, iron, are modified when they become magnetically coupled to the orbits of other atoms and groups of atoms.

It is true that forces of the required order of magnitude are not obtained when we assume that the orbits belonging to different atoms are limited, in their approach, to the accepted atomic distances. However, the phenomenon of ferromagnetism itself leads us to expect that the particular group of orbits responsible for the paramagnetism of the metals of the iron group, the 3_3 orbits of Bohr, are suceptible of being orientated with reference to the other orbits of the atom. If one or more of these orbits can be assumed to be shifted and if, further, they can be assumed to interpenetrate a system of orbits attached to another atom, the approach may be much closer than atomic distances. The orbits may, in fact, actually coalesce and become coplanar. Whatever the nature of the Werner coordination forces leading to the formation of complex salts, it seems to us that a further pursuit along the lines followed in our work and as described in this paper should ultimately lead to a detailed understanding of them. We find evidence of these forces everywhere in chemistry and more generally in nature. But their origin and nature are not yet clear.

[16] Lewis, G. N., Valence and the structure of atoms and molecules, New York, 1923.

STUDIES OF AUTOLYSIS.

XI. RELATION OF THE ISOELECTRIC POINT TO DIGESTIBILITY.

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In previous papers we have shown that liver proteins become increasingly available for autolysis with increasing increments of acid added to the brei (1–4). This increase of digestion, as measured by amino acid production, is apparently caused by an increase of substratum the hydrolysis of which the liver enzymes can catalyze. The data thus far collected do not negate increased enzymic activity with increased H ion concentration, but they do not definitely show it. The relation between acid added and equilibrium attained is apparently a linear one and we have attempted to explain the phenomenon on the basis of a protein-acid combination which constitutes the actual substratum in the autolysis. The more acid added to a digest the more substratum is produced from the non-digestible base-proteins of the cells, and the more amino acids produced, therefore, when final equilibrium is attained.

In some of our previous papers we have noted also that certain foreign proteins, like peptone and gelatin, are substrata at the H ion levels regularly reached by control liver digests—that is, about pH 6. Other proteins, like egg albumin, are not substrata under these conditions, and may even inhibit autolysis (1, 5, 6, 7,). When sufficient strong acid is added egg albumin is found to digest, while the addition of a weak acid or a salt producing a small increase of H ion ($MnCl_2$) fails to convert the albumin into available substratum. These observations, together with the known changes in properties on either side of the isoelectric point, have suggested that the isoelectric point of a protein may determine its availability.

The following experiments were carried out, therefore, with a view to determine whether or not this is the case.

EXPERIMENTAL PART.

A series of foreign proteins has been selected representing a rather wide range of isoelectric values. They were carefully purified and added to control liver breis brought to known pH levels. pH measurements were made by the potentiometer method using a Clark rocking electrode chamber.

TABLE I.

Data Used in Setting up Liver Digests of Edestin.

Titration data.				Digests.			
Edestin (25 cc.).		Liver brei (50 cc.).		Condition.	HCl	pH calculated.	pH determined.
pH	HCl	pH	HCl				
	cc.		cc.		cc.		
6.73		6.66		Control.	0.40	6.5	6.48
6.59	0.20	5.71	3.40	"	2.10	6.0	5.95
5.84	1.00	5.31	5.85	"	4.60	5.5	5.55
5.54	2.00	4.89	8.99	"	8.10	5.0	5.10
4.33	5.00	4.52	13.25	"	13.40	4.5	4.62
3.29	9.95	4.00	20.75	"	20.80	4.0	4.14
1.97	14.95	3.64	29.3	" + edestin.	0.60	6.5	6.53
				" + "	2.95	6.0	6.04
				" + "	6.45	5.5	5.62
				" + "	11.25	5.0	5.08
				" + "	17.90	4.5	4.65
				" + "	26.85	4.0	4.22
				" + "	38.00	3.5	3.72

The pH levels of the digest were controlled by constructing pH titration curves for liver brei and foreign protein separately. It was assumed that the combined buffering value of the liver and foreign protein was equal to the sum of each taken separately. Actual determination showed that for all practical purposes the assumption was correct. As an example, in Table I are given the data by which the edestin and liver digests were made up. Though pH changes occur during digestion, it was found that in control and liver-protein mixtures, the pH changes were of the same magnitude and in the same direction. (Tables I and II.)

The same liver brei was used for constructing the pH-titration curves and setting up the digests. The application of a pH-titration curve of one liver brei to another brei is approximately accurate as the individual livers do not vary greatly in buffering power. In these experiments the same liver was used for deter-

TABLE II.

Typical Changes in H Ion Concentration during Autolysis.

No.	Condition.	pH data.		
		0 days.	1 day.	10 days.
1	Control.	6.5	6.35	6.47
2	" + serum globulin.	6.5	6.5	6.53
3	" + " albumin.	6.5	6.45	6.55
4	" + edestin.	6.5	6.58	6.68
5	"	6.0	6.17	6.3
6	" + serum globulin.	6.0	6.27	6.42
7	" + " albumin.	6.0	6.22	6.44
8	" + edestin.	6.0	6.32	6.57
9	"	5.5	5.84	6.07
10	" + serum globulin.	5.5	5.94	6.19
11	" + " albumin.	5.5	5.84	6.12
12	" + edestin.	5.5	5.87	6.14
13	"	5.0	5.27	5.53
14	" + serum globulin.	5.0	5.27	5.64
15	" + " albumin.	5.0	5.27	5.54
16	" + edestin.	5.0	5.27	5.46
17	"	4.5	4.77	4.76
18	" + serum globulin.	4.5	4.73	4.78
19	" + " albumin.	4.5	4.73	4.76
20	" + edestin.	4.5	4.80	4.78
21	"	4.0	4.28	4.34
22	" + serum globulin.	4.0	4.28	4.38
23	" + " albumin.	4.0	4.28	4.39
24	" + edestin.	4.0	4.36	4.53
25	"	3.5	3.90	4.01
26	" + serum globulin.	3.5	3.90	4.00
27	" + " albumin.	3.5	3.95	3.98
28	" + edestin.	3.5	3.98	3.99

mining the buffer values, and in studying the digestion of added foreign proteins. The digests of one experiment are comparable among themselves at all times, and any differences in digestion can be referred to the foreign protein itself.

An increased production of amino acid in the liver-protein mixtures over the liver alone at the same pH is to be interpreted as expressive of the digestion of the foreign protein. The pH value at which the foreign protein begins to yield unmistakeable increases of amino acid we have designated the "critical point," and in the following series of experiments it will be seen that each protein tried has a critical point that is characteristic of it and which in fact approximates very closely its isoelectric point. As would

TABLE III.

Effect of Edestin on Autolysis.

No.	Condition.	Initial pH.	0.20 N amino acid per 25 cc. filtrate. 0 days.	1 day.	3 days.	5 days.	11 days.	27 days.	Net gain.	Digestion edestin.
			cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	Control.	6.5	0.35	0.62	0.87	1.00	1.20	1.32	0.97	
2	" + edestin.	6.5	0.35	0.75	0.97	1.10	1.30	1.55	1.20	0.23
3	"	6.0		0.84	1.04	1.10	1.45	1.65	1.30	
4	" + "	6.0		0.88	1.10	1.20	1.63	1.85	1.50	0.20
5	"	5.5		1.00	1.15	1.40	1.60	1.90	1.55	
6	" + "	5.5		1.20	1.35	1.70	2.00	2.30	1.95	0.40
7	"	5.0		1.05	1.30	1.70	1.90	2.15	1.80	
8	" + "	5.0		1.25	1.90	2.30	2.70	2.95	2.60	0.80
9	"	4.5		1.00	1.55	1.70	2.05	2.35	2.00	
10	" + "	4.5		1.35	2.10	2.70	2.95	3.35	2.00	1.00
11	"	4.0		1.12	1.55	1.70	1.95	2.35	2.00	
12	" + "	4.0		1.60	2.00	2.60	2.50	2.80	2.45	0.45
13	"	3.5		1.15	1.60	1.60	1.85	2.10	1.75	
14	" + "	3.5		1.65	2.15	2.15(?)	2.50	2.70	2.35	0.60

Total available amino N per 25 cc. filtrate.

In control.....15.41 mg.

" " + edestin.....20.90 "

be expected the critical point is not one at which a sudden or profound change occurs—it cannot be designated exactly—but it can be described as occurring at an approximate pH value.

Digestion has been determined as in previous studies of autolysis by precipitating the proteins with trichloroacetic acid and making a formol titration of an aliquot of the protein-free filtrate. This represents amino acids and ammonium salts, and thus ex-

presses final cleavage. Primary cleavage has been estimated by colorimetric determination of the tyrosine complex by the method of Folin and Denis (3, 8), also on the trichloroacetic acid filtrates. It was found that as a general thing the tyrosine determination paralleled the amino acid determination. Therefore, these data have not been included.

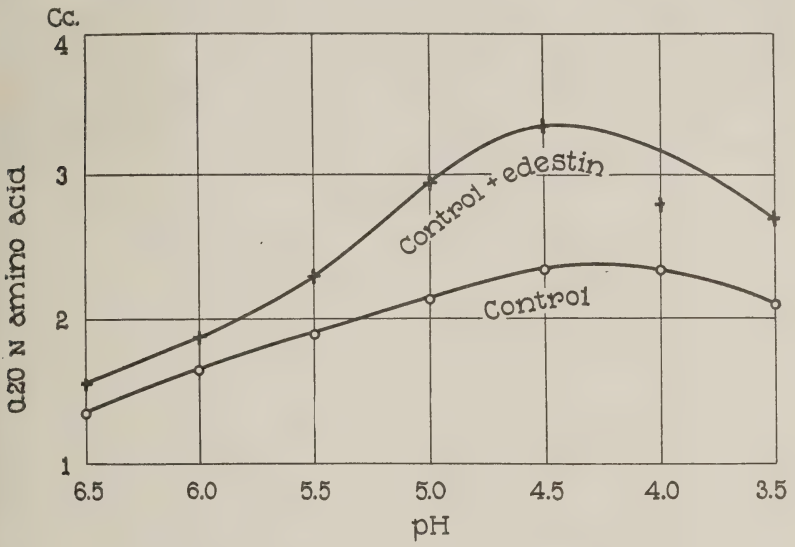

Fig. 1. Effect of edestin on autolysis (27 days autolysis).

Edestin.

Edestin was prepared by extracting ground hemp-seed with gasoline and ether to remove the fat. The residue, rapidly air-dried, was then extracted with 10 per cent NaCl, filtered, and the filtrate dialyzed against tap water. The precipitated edestin was filtered, redissolved with 10 per cent NaCl, and again dialyzed. The finely divided edestin was used in the form of a suspension. (Table III.)

At pH 6.5, edestin is slightly digested. With increasing acidity, increasing amounts of edestin are hydrolyzed, with an optimum at pH 4.5. The slight digestion of edestin near neutrality is similar to the behavior of the liver brei itself. Extrapolation of the curve of Fig. 1 indicates that the critical point of edestin is in the neighborhood of pH 7.0.

Egg White.

Strained, undiluted egg white was used in setting up digests at pH levels differing from each other by 0.5. (Table IV and Fig. 2.)

The data show, first, that the inhibiting effect of egg white on autolysis tends to disappear between pH 5.5 to 5.0; secondly, the inhibition does not appear to be permanent, though this result is not a constant finding. No marked hydrolysis of egg white appears to occur at acidities lower than pH 5.0. Attempts were made to determine the critical point of egg white more accurately, but were found to be impossible because of the comparatively rough analytical methods and probably also the variation in the

TABLE IV.

Relation of H Ion Concentration to Inhibition of Autolysis.

No.	Condition.	Initial pH.	0.20 N amino acid per 25 cc. filtrate.					
			0 days.	2 days.	4 days.	7 days.	25 days.	Net gain.
			cc.	cc.	cc.	cc.	cc.	cc.
1	Control.	6.52	0.37	1.80	2.30	2.20	2.50	2.10
2	" + egg white.	6.52	0.35	1.65	1.80	2.15	2.55	2.15
3	"	6.00	0.35	2.35	2.70	2.80	3.10	2.75
4	" + " "	6.00	0.42	2.25	2.35	2.70	3.20	2.80
5	"	5.50	0.33	2.60	3.00	3.40	3.70	3.35
6	" + " "	5.50	0.33	2.60	2.90	3.35	3.90	3.55
7	"	5.00	0.37	2.80	3.35	3.70	4.90	4.55
8	" + " "	5.00	0.33	2.75	3.95	4.45	5.50	5.15
9	"	4.50	0.40	2.85	3.50	4.10	5.20	4.80
10	" + " "	4.50	0.35	2.85	4.20	5.35	7.30	6.95

autolytic activity of different livers. The effect of a change in pH of 0.1 cannot be satisfactorily measured. However, the data seem to warrant the conclusion that for egg white the critical point is about pH 5.5 to 5.3. In the interpretation of the curves, it should be borne in mind that digestion has gone on until equilibrium is approximated. The upward trend of the control curves show the well known increase of amino acids produced at increased H ion levels. In the case where edestin is present it is evident that both edestin and liver proteins are increasingly available with rising H ion, up to pH 4.5. Where egg white is present, digestion

is definitely inhibited up to pH 5.5, at which level egg white no longer inhibits, but does not contribute anything to the mass of substratum. At higher H ion levels, egg proteins increase the mass of substratum. Between pH 5.5 and 5.0 the greater part of this added protein becomes substratum in the digest.

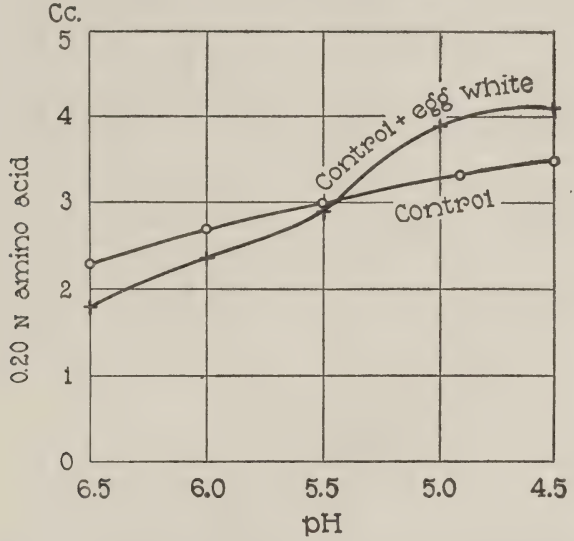

FIG. 2. Effect of egg white on autolysis (4 days autolysis).

Ovalbumin.

Dialyzed, recrystallized ovalbumin was used. (Tables V and VI and Figs. 3 and 4.)

The data reveal the following tendencies: (1) inhibition by ovalbumin during the first 16 hours in the pH range 5.5 to 4.9; (2) the inhibitory effect does not appear to influence the final equilibrium; and (3) no marked hydrolysis of the albumin at acidities lower than pH 5.0 (though this appears to be somewhat contradicted by the tyrosine determinations). The critical point of ovalbumin would appear to be about pH 5.0.

TABLE V.

Digestion of Egg Albumin by the Liver Enzymes.

No.	Condition.	Initial pH.	0.20 N amino acid per 25 cc. filtrate.						
			0 days.	½ day.	2 days.	5 days.	18 days.	Net gain.	Digestion albumin.
			cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
1	Control.	5.5	0.40	2.00	2.90	3.10	3.85	3.45	
2	" + albumin.	5.5	0.40	1.50	2.60	3.00	3.65	3.25	−0.20
3	"	5.20		2.00	2.95	3.40	4.60	4.20	
4	" + "	5.2		1.60	2.90	3.70	4.40	4.00	−0.20
5	"	5.0		1.95	3.00	4.00	5.10	4.70	
6	" + "	5.0		1.80	3.05	4.00	5.20	4.80	0.10
7	"	4.9		1.80	3.15	4.30	5.25	4.85	
8	" + "	4.9		1.80	3.10	4.40	5.50	5.10	0.25
9	"	4.8		1.90	3.10	4.50	5.20	4.80	
10	" + "	4.8		1.90	3.50	4.50	6.05	5.65	0.85
11	"	4.7		2.00	3.50	4.40	5.50	5.10	
12	" + "	4.7		2.00	3.50	4.75	6.35	5.95	0.85
13	"	4.5		2.15	3.60	4.60	5.20	4.80	
14	" + "	4.5		2.10	3.60	4.60	6.10	5.70	0.90
15	"	4.0		2.20	3.60	4.50	5.40	5.00	
16	" + "	4.0		2.30	3.80	4.80	6.10	5.70	0.70

TABLE VI.

Tyrosine Liberation in the Digests of Table V.

No.	Condition.	Initial pH.	Tyrosine per 5 cc. filtrate.						
			0 days.	½ day.	2 days.	5 days.	18 days.	Net gain.	Digestion albumin.
			mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Control.	5.50	0.298	1.072	1.476	1.762	2.090	1.795	
2	" + albumin.	5.50	0.292	0.992	1.300	1.610	1.945	1.650	−0.145
3	"	5.20	0.295	1.030	1.560	1.900	2.250	1.955	
4	" + "	5.20		1.025	1.520	1.900	2.340	2.045	0.090
5	"	5.00		1.107	1.638	2.065	2.475	2.180	
6	" + "	5.00		1.087	1.557	2.065	2.675	2.380	0.200
7	"	4.90		1.102	1.648	2.140	2.520	2.225	
8	" + "	4.90		1.132	1.640	2.230	2.740	2.445	0.220
9	"	4.80		1.137	1.660	2.170	2.570	2.275	
10	" + "	4.80		1.198	1.820	2.405	2.860	2.565	0.290
11	"	4.70		1.233	1.880	2.350	2.750	2.455	
12	" + "	4.70		1.272	1.905	2.450	3.080	2.785	0.330
13	"	4.50		1.300	1.940	2.490	2.850	2.555	
14	" + "	4.50		1.325	2.000	2.610	3.260	2.965	0.410
15	"	4.00		1.389	2.200	2.610	2.740	2.445	
16	" + "	4.00		1.490	2.340	2.785	3.370	3.075	0.630

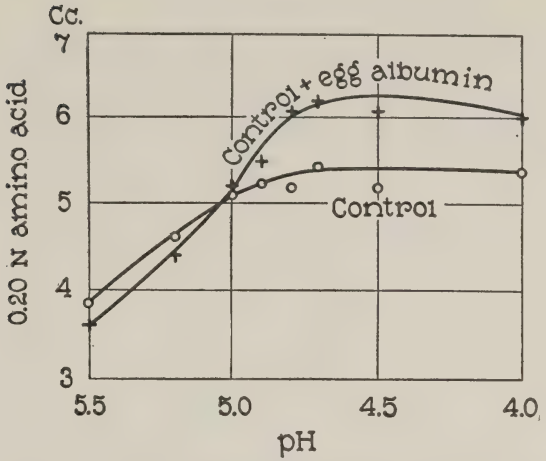

Fig. 3. Effect of egg albumin on autolysis (18 days autolysis).

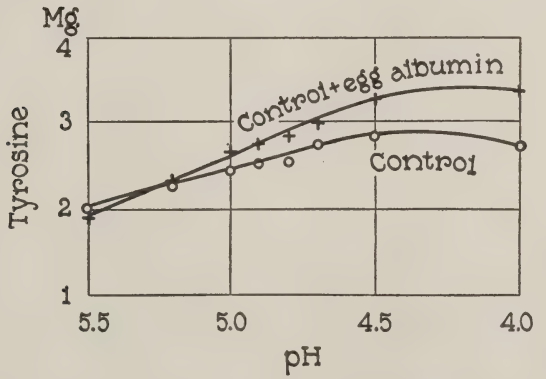

Fig. 4. Effect of egg albumin on autolysis (18 days autolysis).

Ovoglobulin.

Ovoglobulin was prepared by reprecipitating several times with $(NH_4)_2SO_4$ at half saturation, then dialyzing thoroughly. The globulin was used in the form of a suspension. (Table VII.)

Ovoglobulin inhibits autolysis between pH 6.0 and 5.0. At pH 5.0, some of the protein becomes available substratum for the liver enzymes. It would appear that the critical point is approximately pH 5.3.

TABLE VII.

Digestion of Egg Globulin by the Liver Enzymes.

No.	Condition.	Initial pH.	0.20 N amino acid per 25 cc. filtrate.			
			0 days.	10 days.	Net gain.	Digestion protein.
			cc.	*cc.*	*cc.*	*cc.*
1	Control.	6.0	0.43	1.72	1.29	
2	" + globulin.	6.0	0.43	1.15	0.70	−0.59
3	"	5.5	0.43	1.95	1.52	
4	" + "	5.5	0.43	1.68	1.25	−0.27
5	"	5.0	0.43	2.47	2.05	
6	" + "	5.0	0.43	2.57	2.15	0.10
7	"	4.5	0.43	2.50	2.10	
8	" + "	4.5	0.43	2.83	2.40	0.30
9	"	4.0	0.43	2.35	1.90	
10	" + "	4.0	0.43	2.83	2.40	0.30

Total available amino N per 25 cc. filtrate.

In control.....16.35 mg.

" " + globulin.....38.05 "

TABLE VIII.

Digestion of Serum Globulin and Serum Albumin by the Liver Enzymes.

No.	Condition.	Initial pH.	0.20 N amino acid per 25 cc. filtrate.			
			0 days.	10 days.	Net gain.	Digestion protein.
			cc.	*cc.*	*cc.*	*cc.*
1	Control.	5.5	0.43	1.95	1.52	
2	" + globulin.	5.5	0.43	1.55	1.12	−0.40
3	" + albumin.	5.5	0.43	1.52	1.10	−0.42
4	"	5.0		2.47	2.05	
5	" + globulin.	5.0		1.98	1.55	−0.50
6	" + albumin.	5.0		2.60	2.15	0.10
7	"	4.5		2.50	2.10	
8	" + globulin.	4.5		3.00	2.57	0.47
9	" + albumin.	4.5		3.25	2.80	0.70
10	"	4.0		2.35	1.90	
11	" + globulin.	4.0		3.05	2.62	0.72
12	" + albumin.	4.0		3.00	2.57	0.67

Total available amino N per 25 cc. filtrate.

In control.....16.35 mg.

" " + globulin.....22.75 "

" " + albumin.....30.20 "

Serum Albumin and Serum Globulin.

Serum albumin was prepared by reprecipitation with $(NH_4)_2SO_4$ (saturated) in weakly acid medium and thorough dialysis. Serum globulin was prepared by reprecipitation with $(NH_4)_2SO_4$ (half saturation) and thorough dialysis. Both proteins were used in small amounts. (Table VIII and Fig. 5.)

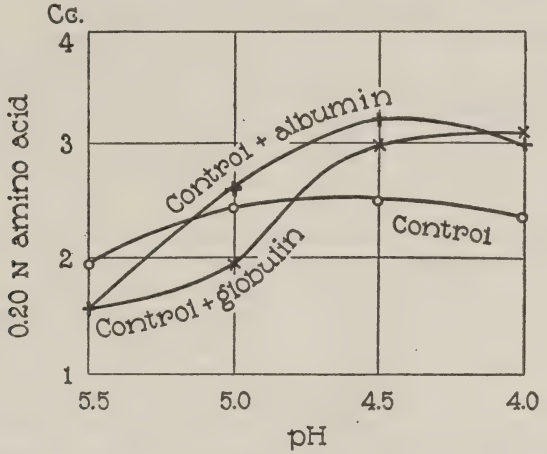

FIG. 5. Effect of serum albumin and globulin on autolysis (10 days autolysis).

The data show inhibition of autolysis by globulin at acidities lower than pH 4.8 and by albumin at acidities lower than pH 5.0. Digestion of albumin begins about pH 5.0 and of globulin about pH 4.8. The greater inhibitory power of globulin is to be noted, even though it is present in smaller amounts than albumin.

The critical point of serum globulin appears to be about pH 4.8; that of serum albumin about pH 5.0.

Serum.

A comparison of the above experiment with the preceding is interesting. Serum inhibits autolysis at H ion concentrations below pH 4.6, above which it appears to digest. This is about the same pH at which the inhibitory effect of serum globulin disappears and suggests that the behavior of serum is determined by the globulin. (See Table IX.)

If the critical points of the proteins and their isoelectric points (9) are compared, a striking relationship is evident, as seen in Table X. The critical point is seen to be at an H ion concentration slightly below the isoelectric point of the protein involved. These findings we believe to be direct evidence that the proteins are in

TABLE IX.

Effect of Serum on Liver Autolysis.

No.	Condition.	Initial pH.	0.20 N amino acid per 25 cc. filtrate.								
			0 days.	½ day.	1 day.	2 days.	4 days.	7 days.	12 days.	Net gain.	Digestion protein.
			cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	Control.	6.50	0.30	1.30	1.62	1.80	1.95	2.30	2.35	2.05	
2	" + serum.	6.50	0.30	0.90	1.00	1.15	1.35	1.50	1.70	1.40	−0.65
3	"	5.50		1.65	2.15	2.85	3.55	4.00	4.00	3.70	
4	" + "	5.50		1.35	1.65	2.05	2.20	2.80	2.70	2.40	−1.30
5	"	5.00		1.90	2.50	3.00	3.70	4.10	4.30	4.00	
6	" + "	5.00		1.70	1.90	2.70	3.00	3.35	3.30	3.00	−1.00
7	"	4.50		2.00	2.65	3.00	3.70	3.90	4.45	4.15	
8	" + "	4.50		1.95	2.70	3.15	4.20	4.55	5.05	4.75	0.60

Total available amino N per 25 cc. filtrate.

In control.....35.95 mg.

" " + serum.....39.95 "

TABLE X.

Protein.	Isoelectric point.	Critical point.
	pH	*pH*
Egg albumin.	4.8	5.0
" globulin.	5.2	5.3
" white.		5.5–5.3
Serum.		4.6
" globulin.	4.4	4.8
" albumin.	4.7	5.0
Edestin.	6.9	7.0 (?)

the form of acid salts when digested by the liver proteases. The inhibitory effect of the albumin and globulin appears to be associated with the formation of a stable protein-enzyme complex which does not proceed to cleavage, but on the contrary, removes

enzymes from the field of action. Further discussion and interpretation of these results will be postponed for a following paper.

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INSULIN AND PHLORHIZIN GLYCOSURIA.

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The primary aim of these experiments was to determine whether or not insulin has an effect on the glycosuria of a dog made diabetic by phlorhizin. Incidentally they permitted observation of the effects of certain diets on the metabolism in phlorhizin diabetes that have a clinical bearing.

EXPERIMENTAL.

Experiment 1.—A female dog weighing 9.6 kilos received phlorhizin, 1 gm. suspended in 10 cc. of olive oil subcutaneously once every 24 hours, also 0.38 cc. of epinephrine (Parke, Davis and Co. adrenalin ampoules, 1–1,000) once every 4 hours as described by Sansum and Woodyatt (1). The animal received water but no food. The urine was collected by catheter and the bladder irrigated every 8 hours. The urine for each 8 hour period was made up to a standard volume of 1,000 cc. and analyzed in duplicate for sugar by the Folin-Berglund (2) and Benedict-Osterberg (3) methods, and for nitrogen by Kjeldahl. After a preliminary 16 hour period the D:N ratio had fallen to 3.65 and the figures by 8 hour periods then ran as follows:

Period.	Glucose.	Nitrogen.	D : N
1	12.0	3.3	3.64
2	12.8	3.5	3.66
3	11.7	3.2	3.66
Average.....	12.2	3.3	3.65

The epinephrine was then omitted for 8 hours and then resumed for one period.

Period.	Glucose.	Nitrogen.	D : N
4	10.5	3.4	3.09
5	11.3	3.2	3.53

At this stage it is apparent that epinephrine still had the power to increase slightly the D:N ratio, so the animal showed evidences of still retaining a trace of glycogen, but owing to the slightness of the difference, the dog's condition, and the specific purpose of the experiment, it was deemed expedient to proceed notwithstanding the fact that it was not proved that the constant ratio of the fore periods was as low as it might be made. Beginning with Period 6 the epinephrine was permanently discontinued. Phlorhizin, however, was continued as before, and 33 cc. of a milk and cream mixture were then given by mouth every 4 hours with the intention of supplying approximately as much fat as the dog was catabolizing in fasting. *The food mixture* contained, by analysis, carbohydrate 3.3, protein 2.75, and fat 12.18 per cent, and the ration provided carbohydrate 2.2, protein 1.83, and fat 8.12 gm. per 8 hours. The caloric value was 30 calories per kilo per 24 hours. For the first period on this program the figures were:

Period.	Glucose.	Nitrogen.	D : N
6	9.3	2.2	4.23

but for the succeeding four 8 hour periods they were as follows:

Period.	Glucose.	Nitrogen.	D : N
7	11.9	3.0	3.97
8	13.2	4.1	3.22
9	12.1	3.9	3.11
10	13.3	3.5	3.80
Average.....	12.6	3.6	3.53

It will be noted that, except for the transitional Period 6, the feedings left the figures essentially *in statu quo ante*. At the start of the next period the animal received a subcutaneous injection of insulin, 20 units (iletin, Lilly, No. 746252).

Period.	Glucose.	Nitrogen.	D : N
11	9.6	2.6	3.69

Thus the glucose was reduced by 3 gm. and the nitrogen by 1. In this period, 6 hours after the insulin injection, the dog went into a convulsion but recovered without treatment and two after periods, with the feedings and the phlorhizin as before, but with no insulin, ran as follows:

Period.	Glucose.	Nitrogen.	D : N
12	11.8	2.8	4.22
13	11.9	3.1	3.84
Average.....	11.9	3.0	4.03

Thus the glucose excretion virtually resumed its former level, but the nitrogen lagged and owing to the relative lowering of the nitrogen the D:N ratio was higher than before. This might make it appear that "extra" sugar had been excreted, but such a conclusion would not be confirmed by any absolute increase of the glucose output. In the final three periods phlorhizin and feedings were continued and epinephrine was resumed to ascertain whether or not there had been a demonstrable deposition of glycogen during the foregoing periods of feeding.

Period.	Glucose.	Nitrogen.	D : N
14	12.3	3.3	3.73
15	13.5	3.2	4.22
16	12.7	3.1	4.10
Average.....	12.8	3.2	4.02

It will be seen at this stage of the experiment that epinephrine had no appreciable effect on the rate of sugar excretion. The experiment was then stopped with the dog in good condition after continuous observations for 144 hours. The temperature range was 99.6 – 101.6°F.

Experiment 2.—A female dog weighing 7.9 kilos was used. The phlorhizinization, epinephrinization, urine collections, and analyses were conducted as before. At the end of 32 hours of fasting a constant D:N ratio of 3.3 was established, *unaffected by the omission of ephinephrine,* although the effects of resuming the epinephrine were not tested. It would seem that the dog showed no positive evidence of retaining glycogen. The results for the last two 8 hour fore periods were as follows:

Period.	Glucose.	Nitrogen.	D : N
1	11.1	3.3	3.36
2	10.4	3.2	3.25
Average.....	10.8	3.3	3.31

At this stage phlorhizin was continued as before, epinephrine was stopped, and feedings were begun with 100 cc. of a milk and cream mixture once

every 8 hours prepared in the same way as that used in the previous experiment although more of it was given. The next three periods ran as follows:

Period.	Glucose.	Nitrogen.	D : N
3	12.9	3.1	4.17
4	11.8	2.5	4.22
5	10.4	2.8	4.16
Average.....	11.7	2.8	4.18

All other conditions remaining the same, the feedings were now doubled, 200 cc. of the mixture being given every 8 hours until the end of the experiment. This was attended by some looseness of the bowels with fatty stools, and the urinary figures showed some irregularity of the glucose excretion.

Period.	Glucose.	Nitrogen.	D : N
6	12.2	2.7	4.52
7	8.5	2.2	3.87
8	9.7	2.1	4.62
9	12.4	2.0	6.20
10	9.6	1.9	5.05
11	11.8	1.8	6.56
Average.....	10.7	2.1	5.14

5 units of insulin (iletin, Lilly, No. 746251) were then given subcutaneously.

Period.	Glucose.	Nitrogen.	D:N
12	9.6	1.5	6.40

Then for 8 hours no insulin was given.

Period.	Glucose.	Nitrogen.	D:N
13	10.4	1.7	6.12

20 units of the same preparation of insulin were then given.

Period.	Glucose.	Nitrogen.	D:N
14	8.7	1.5	5.80

The next period was without insulin.

Period.	Glucose.	Nitrogen.	D:N
15	9.8	1.7	5.76.

The experiment was then stopped, the dog having retained her weight, and remained afebrile and in much better condition than at the start of the feedings.

COMMENTS ON THE EXPERIMENTS.

1. Feedings.—The purpose of the feedings was to maintain the nutrition of the animal for a sufficient length of time with a constant glycosuria to permit the desired observations on the effects of insulin with satisfactory controls.

In the first experiment approximately 30 calories per kilo per 24 hours were supplied by the food. The dog remained at virtually the same weight for 6 days although not in nitrogen equilibrium at any time. The total glucose and nitrogen excretions and the ratio of D:N were practically unaffected by the feedings.

In the second experiment the feedings supplied about 60 calories per kilo per 24 hours in Periods 3, 4, and 5, and 120 calories per kilo per 24 hours in Periods 6 to 15, inclusive. It is interesting to note that these feedings were attended by the following phenomena.

1. No appreciable increase of the total glucose excretion over that of the fasting periods.

2. Reduction of the protein metabolism (as indicated by the urinary nitrogen) from 8.4 gm. per kilo per 24 hours in fasting (Periods 1 and 2) to 4.5 gm. per kilo per 24 hours after feeding alone (Period 11), a decrease of nearly 50 per cent.

3. An arrest of the progressive weight loss.

4. A notable improvement in the general condition.

These observations show strikingly how the feeding of a diet sufficiently high in fat may greatly reduce the protein metabolism in the case of a diabetic organism impoverished in body fat, but in which the protein is not as yet correspondingly depleted. In this case the rapid depletion of the body fat had led to a great increase of the protein metabolism which was reduced by the

feedings. The analogous phenomenon in human diabetics has been described by Woodyatt (4). The rationale of the clinical use of fat replacement diets to reduce the protein breakdown and hence the endogenous supply of glucose from protein is thus illustrated.

2. Insulin.—The effect of the insulin injections on the absolute glucose excretion is summarized below.

Experiment.	Insulin given.	Average excretion in fore periods.	Excretion in insulin period.	Average excretion in after periods.	Absolute depression.*	Sugar per unit of insulin.
	units	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	20	12.6	9.6	11.9	2.7	0.1
2	5	10.7	9.6	10.4	1.0	0.2
2	20	10.7	8.7	9.8	1.6	0.1

*Below the average of the fore and after periods.

It appears that the insulin caused a slight but measurable absolute depression of the total sugar excretion. This was accompanied by a definite depression of the nitrogen output in one instance and by slight depressions in the other two. The glucose returned promptly to approximately its former level while the nitrogen lagged.

3. The D:N Ratio and "Extra" Glucose.—In Experiment 1, Periods 7 to 10, inclusive, in which the dog received feedings but no epinephrine, the average total excretions of sugar and nitrogen and the ratios between them remained almost exactly as they had been in the fore periods, Nos. 1 to 3 inclusive, in which the dog fasted and received epinephrine. In other words, the milk and cream feedings had no appreciable effect on the outputs of sugar and nitrogen, the metabolism in the feeding periods remaining the same as it was in fasting. It would seem that the fat and protein of the diet had acted simply as replacements for corresponding quantities of tissue fat and protein catabolized in fasting. It will be noted, however, that the diet contained 2.2 gm. of carbohydrate in the form of lactose per 8 hour period, and that this failed to register in the urine as "extra" glucose when the 3.65 ratio found in the fore periods was used as the basis of calculation for "extra" sugar in these

latter periods. The question arises as to what became of the sugar of the diet. The first possibility to suggest itself might be that of storage as glycogen. If stored as glycogen it might be recovered by a resumption of the epinephrine injections. Epinephrine injections were accordingly instituted in the last three periods of the experiment. In these periods (Nos. 14, 15, and 16) under epinephrine, however, only 3.46 gm. in all of "extra" sugar appeared, calculated as the sugar excreted in excess of total nitrogen times 3.65 (the average D:N ratio found in the fore periods). This would account for but 58 per cent of the lactose contained in the feedings for the last three periods alone, and would not account for any of the carbohydrate missed during the eight preceding periods for which the total amount of carbohydrate contained in the feedings was 17.6 gm. All this disappeared permanently. This would necessitate one of two conclusions, either (*a*) that this lactose was destroyed in the body, or (*b*) that the original ratio of 3.65 obtained in the fore periods was not strictly applicable in the feeding periods to indicate the amount of glucose actually formed from the protein catabolized in these particular periods.

In continued fasting experiments the ratio may gradually fall in later periods to 3.3, 3.0, or 2.8. If it were assumed that the glucose formed from the protein actually catabolized in those particular periods had equalled 2.8 instead of 3.65 times the nitrogen, then "extra" sugar estimated on the basis of the 2.8 ratio would leave for these periods no lactose unaccounted for. In any event, the experiment taken by itself does not permit of a final conclusion as to whether the missing carbohydrate was actually destroyed or whether it was simply lost from view owing to the method of calculating. The experiment shows the necessity of adequate controls and caution in the interpretation of results. It also illustrates strikingly how the feeding of a replacement diet may leave the metabolism in the same status that it was during fasting.

In Experiment 2, Periods 6 to 11, inclusive, with feedings but no epinephrine, the aggregate output of glucose was 64.2 gm. and of nitrogen 12.7 gm. Assigning the value of 3.3 to D:N, the "extra" glucose for Periods 6 to 11 was 22.3 gm. In the same periods the glucose supply from the carbohydrate of the

diet was 39.6 gm. Thus only 56 per cent of the carbohydrate ingested is accounted for as "extra" sugar. How much more "extra" glucose might possibly have been recovered by the use of epinephrine to discharge any accumulation of glycogen was not determined, but the other dog showed no glycogen accumulation. In this experiment the use of an hypothetical 2.8 ratio for the calculation of "extra" sugar would still account for but 72 per cent of the lactose of the food. It seems, therefore, that some lactose must have been utilized.

CONCLUSIONS DRAWN FROM THE EXPERIMENTS.

Three injections of insulin (iletin, Lilly) into phlorhizinized dogs have been followed by measurable but slight depressions of the glucose excretion, accompanied in one instance by a definite depression of the nitrogen elimination and by slight to doubtful depressions in the other two. The absolute depressions of the glycosuria amounted to 2.7, 1.6, and 1.0 gm., corresponding, respectively, with 0.14, 0.08, and 0.20 gm. of sugar per unit of insulin given. These depressions are not over one-half to one-twentieth those that may be obtained in cases of human diabetes mellitus in comparable metabolic states. The slight reductions of the glycosuria accompanied by depressions of the nitrogen elimination following hypodermic injections of iletin into highly sensitive phlorhizinized dogs suggest simple retentions, induced by something contained in the insulin preparation. It would seem theoretically probable that the supplying of an excessive amount of insulin to the phlorhizinized organism could lead to some acceleration of the rate of glucose oxidation or storage in the tissues and thus through the physiological action of insulin itself account for a limited depression of the sugar elimination. On the other hand, it would seem highly improbable that insulin exerts any lessening, annulling, or antidoting action on the physiological action of phlorhizin itself, because in all the experiments the total reduction of the glycosuria was slight, notwithstanding the fact that one of the dogs received enough insulin to produce what appeared to be typical hypoglycemic convulsions. No animal even approached the non-glycosuric status after receiving insulin and no constant change was observed in the D:N ratio. The writer therefore inclines to the

view that the injection of iletin may have caused some slight increase of the glucose utilization coupled with injuries to the kidneys or to the tissues at large resulting in retentions. To determine whether this is the true explanation and if so whether the retention has been caused by the insulin itself, the preservative in the preparation, or impurities that it is not as yet possible to separate from insulin, could only be stated after conducting a sufficient series of adequately controlled experiments.

OTHER REPORTS IN THE LITERATURE.

The Experiments of Nash.—Nash (5) reports two experiments on fasting completely phlorhizinized dogs, one of which (that on Dog 3[1]) may be taken as representative. He records results for three 24 hour periods in the second of which 10 units of iletin were given. He states regarding the glycosuria:[2] "Following the injection of iletin there occurred a marked disturbance of the D:N ratios. With the falling blood sugar there was a sharply diminished excretion of sugar in the urine. The hourly rate decreased from 1.82 gm. .....to about 0.90 gm..... Then occurs a sharp rebound which extends temporarily above the average control rate. Coincidently with the fall in sugar elimination, the rate of nitrogen excretion also drops off."

These remarks refer to relative changes occurring in short intervals of time and the aggregate changes obtained per 8 or 24 hours agree with those here reported. The data taken by periods of 8 hours show a maximum diminution in glycosuria of 2.45 gm. below the average of the fore and after periods, corresponding with 0.25 gm. of glucose per unit of insulin given. As noted by Nash, the greatest relative depression of the glycosuria appears to have developed within an hour or two after the injection and to have been followed soon after by a corresponding increase of the output, suggesting a retention. Concerning the observed depressions of the nitrogen outputs following insulin administrations, Nash suggests that they may be due to a sparing action of retained glucose on the protein metabolism rather than to retentions due directly to the same factors that caused the glucose retention, because following the depressions of nitrogen no compensatory increases were observed, and because of the behavior of the D:N ratios. However, conditions capable of causing retentions of glucose are as a rule capable of causing retentions of nitrogen, and there would be perhaps the advantage of simplicity in explaining both directly on the basis of simple retentions plus possibly some slight increased burning of glucose.

The Experiments of Ringer.—Ringer (6) concludes that the injection of insulin into phlorhizinized dogs causes reduction of the glycosuria corresponding with a maximum of 0.95 gm. of glucose per unit of insulin given, also depressions of the protein metabolism and of the ketonuria. He also re-

[1] Nash (5), pp. 456–457.

[2] Nash (5), p. 459.

ports having shown by indirect calorimetry increases of the oxidation and storage of glucose following the injection of insulin. On the basis of all the experiments he proposes the use of phlorhizinized dogs for assaying insulin preparations and advances an hypothesis concerning the action of phlorhizin to the effect that phlorhizin causes glycosuria by interfering with the production of insulin by the pancreas. Without entering into a discussion of the general proposals made by Ringer concerning the nature of the action of phlorhizin, the use of phlorhizinized dogs for the assay of insulin preparations, or of the collateral data on which these proposals are partly based, it may be stated that his conclusion concerning the *degree of depression of the glycosuria* of phlorhizinized dogs following iletin injection rests mainly on seven experiments in which quantitative urinary data were obtained. These may be separated for purposes of discussion into three groups; first, an experiment in which a fasting phlorhizinized dog after two 12 hour fore periods received insulin in the next two periods; second, an experiment in which a phlorhizinized dog after a fore period received 200 gm. of meat with no insulin and in a later period 200 gm. of meat plus 10 units of insulin; and third, five experiments in which different dogs received differing doses of glucose and insulin.

Taking up the several experiments in order; the fasting dog experiment (Dog 2, Table II[3]) shows glucose excretions for the two 12 hour fore periods of 14.2 and 14.9 gm., respectively, or 29.1 gm. for 24 hours. In the first 12 hour insulin period the glucose output was 12.0 gm. and in the next 16.8 gm., which gives for the 24 hour insulin period 28.8 gm.; *i.e.*, no significant difference for the 2 days. The depression of the glycosuria in the first 12 hour insulin period and its compensatory elevation in the second are, moreover, in keeping with the idea of a retention followed later by an elimination of the retained products. These data resemble our own and those of Nash.

The record of the experiment with the meat-fed dog (Dog 5, Table II[3]) shows that in the first 12 hour meat period with no insulin the total sugar excretion was 21.4 gm. and in the 12 hour meat-insulin period 17.5 gm., a difference of 3.9 gm., corresponding with 0.39 gm. per unit of insulin given. This absolute depression is greater than any found in the writer's experiments but as low as any claimed by Ringer. The question naturally arises as to how much difference there would have been between the sugar excretions in the two meat periods if no insulin had been given in the second. The meat feedings were given 36 hours apart. During such an interval the metabolism of a fully phlorhizinized dog has time to change materially, owing to the heavy and continued drainage of its tissue fat and protein. Ringer presents no data to show that two feedings of 200 gm. of meat given to the same fasting dog, one in an early and one in a late period of a phlorhizin run, will necessarily yield exactly the same amount of sugar in the urine. Ringer, moreover, interpreting this experiment by 24 hour periods, finds even a wider difference than that shown by 12 hour periods. Adding

[3] Ringer (6), p. 489.

together the excretions for the 12 hour meat period and the next one, and similarly those of the 12 hour meat-insulin period and the next one, the difference by 24 hour periods is 6.4 gm. or 0.64 gm. of glucose per unit of insulin given. But to group this particular experiment by 24 hour periods leaves it with no after period. It is clear on general principles that at the end of this experiment in which the dog had been totally diabetic for 3 days the animal must have been approaching exhaustion, and as shown by the record of the last 12 hours the glucose was lower than it had been at any time after the first period. The nitrogen was also lower and the D:N ratio had curiously risen to 3.9 which was higher than for any earlier period. In these conditions the data would have to be interpreted with reserve.

The five glucose-insulin experiments (Dogs 11, 4, 1, 3, and 12, Table II[4]) lack adequate controls. One of the glucose animals (Dog 11) received two doses of 40 gm. of glucose 36 hours apart, the second dose accompanied by an injection of 20 units of iletin. In the glucose period 36 gm. of "extra" sugar appeared, or 90 per cent of the dose. In the two 12 hour glucose-insulin periods 28.5 gm. of "extra" sugar appeared. Ringer regards the difference of 7.5 gm. as the measure of the insulin effect, and so states that the 20 units of insulin accounted for 0.38 gm. of glucose per unit. No data are given, however, to show the range of variation of "extra" sugar outputs following two 40 gm. doses of glucose without insulin given 36 hours apart to the same dog. It would seem that part at least of the difference of 7.5 gm. might fall within the limits of error of the method. In the other four glucose experiments four dogs received differing doses of glucose together with insulin. None of these dogs received a control dose of glucose without insulin. Ringer depends wholly for control on the single observation of the "extra" sugar return from the 40 gm. dose in the case of Dog 11, proceeding on the basis that Dogs 4, 1, 3, and 12, with doses of 37, 10 (in two successive periods), 20, and 30 gm. of glucose, respectively, should excrete 90 per cent of each dose, and that the difference between the anticipated 90 per cent excretion and the observed excretion was the measure of the insulin effect.

In this connection Sansum and Woodyatt (7) reported observations on a series of five phlorhizinized dogs receiving single doses of C. P. glucose which excreted as "extra" sugar the following weights and percentages.

Dog	a	given	16	gm.	recovered	13.8	gm.	or	86.3	per cent.
"	b	"	16	"	"	12.1	"	"	75.4	" "
"	c	"	16	"	"	12.0	"	"	74.8	" "
"	d	"	15	"	"	11.0	"	"	73.3	" "
"	e	"	8	"	"	4.2	"	"	52.4	" "
Total		"	71	"	"	53.1	"	"	72.4	" "

All of these five dogs with no insulin excreted less than 90 per cent of the dose of glucose. Had they received insulin and excreted "extra" sugar as above shown, an application of Ringer's interpretation would lead to the

[4] Ringer (6), pp. 488–489.

conclusion that the insulin had reduced the glycosuria by 0.6, 2.3, 2.4, 2.5, and 3.0 gm., respectively. Accordingly it is not evident that Ringer's experiments actually prove diminutions of the glycosuria of the magnitudes claimed.

Conclusions Concerning the Observations of Ringer.—In one experiment on a fasting phlorhizinized dog receiving insulin there was a depression of the glycosuria in the first 12 hours followed by a corresponding elevation in the second with no change in the aggregate sugar output for 24 hours. This result suggests a retention. In all the other experiments the lack of adequate controls makes it impossible to state the degree to which the glycosuria was depressed by the insulin. The diminished tests for acetone bodies and the diminished nitrogen outputs in the urine could also be explained on the basis of retentions with some increase of the glucose oxidation.

The Experiments of Cori.—C. F. Cori (8), working with phlorhizinized rabbits, concludes that insulin injection causes depression of the blood sugar concentration followed by return to the previous level 5 to 6 hours later, also that insulin produces a marked diminution of the sugar excretion. The sugar excretion according to Cori ". . . . may fall to one-sixth of that of the control period." He concludes that "Insulin does not diminish to any larger extent the nitrogen excretion . . . . and does not influence the sugar formation from proteins . . . ." He interprets the diminution of glycosuria as the result of retention. It may be noted that Cori employed 1 hour periods and that the statement that the sugar excretion may fall to one-sixth of that of the control period does not imply greater absolute depressions of the glycosuria than those here reported. Thus for example Table V[5] shows the sugar excretion falling from 0.304 to 0.045 gm. per hour, an absolute difference of 0.259 gm. The greatest aggregate depression of the glycosuria recorded by Cori corresponds with about 0.2 gm. per unit of insulin given.

SUMMARY.

Two experiments are described in which phlorhizinized dogs by the use of uniform milk and cream feedings were kept running steady glucose excretions for 6 days each, finally completing the experiment in excellent physical condition. These dogs were given injections of iletin, three in all, with resultant depressions in the outputs of sugar corresponding with 0.14, 0.20, and 0.08 gm. per unit of insulin given. Following one of the iletin injections the nitrogen output was also definitely diminished for 8 hours, and was perhaps slightly diminished after the other two injections. It is suggested that impairments of elimination

[5] Cori (8), p. 103.

due to toxic effects on the kidneys or the tissues at large following the administration of insulin preparations, coupled possibly with some slight increase of the oxidation of glucose due to insulin itself, would explain the phenomena.

Apart from their bearing on the action of insulin in phlorhizin glycosuria the experiments are of interest in connection with Geelmuyden's recent revival of the conception that epinephrine causes an increased production of sugar from fat (9). The experiments confirm the view that epinephrine has no power to increase the glycosuria of phlorhizinized dogs after precautions have been taken to render them glycogen-free.

The writer expresses his thanks to Dr. R. T. Woodyatt for guidance in the preparation and interpretation of this work.

Technical assistance in the experimental procedure was given by Dr. K. E. Barber.

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SPINACIN, A NEW PROTEIN FROM SPINACH LEAVES.*

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In a recent paper[1] the author described a new method of extracting vacuole and cytoplasmic material from leaf cells which appeared to offer an opportunity for the chemical examination of the cell proteins. By applying this method it has been found possible to isolate a protein or mixture of proteins from the cytoplasm of the spinach leaf in a state of purity such that it does not give the delicate Molisch test for carbohydrates. Considering the complex nature of cytoplasm, and how little is known of its chemistry, the preparation of such a product, without any apparent hydrolytic change, is of importance. The protein appears to be present in the cytoplasm as an anion at a hydrogen ion concentration only slightly lower than that of its isoelectric point; an investigation of its properties, then, may facilitate the chemical interpretation of the reactions observed in living cytoplasm.

EXPERIMENTAL DETAILS.

The leaves of the common spinach, *Spinacia oleracea*, were employed in the experiments undertaken to investigate more

* Seessel Research Fellow, Yale University. The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

The author wishes to express his thanks to Dr. Thomas B. Osborne for his interest in this work and also for much helpful advice and criticism.

[1] Chibnall, A. C., *J. Biol. Chem.*, 1923, lv, 333. In this paper the term "protoplasmic material" was meant to embrace the chloroplasts and other possible inclusions. "Cytoplasmic material" expresses this without ambiguity; it will, therefore, be used in the future in place of the former term. The use of ether to facilitate the extraction of proteins from yeast by Schroeder and others (quoted by Thomas, P., *Ann. Inst. Pasteur*, 1921, xxxv, 43) should have been noted.

fully the ether separation of vacuole and cytoplasmic material described in a recent paper.[1] For the more complete separation of the vacuole material the employment of 0.002 N HCl instead of distilled water had been recommended for washing the pressed residues (before grinding); the object of this being to prevent the solution of the cytoplasmic proteins by changing their hydrogen ion concentration approximately to the isoelectric point. Experience has shown that this precaution is unnecessary; distilled water does not wash out any appreciable quantity of the cytoplasmic proteins, the small amount of protein found in the vacuole extract being different in character from that which can be subsequently obtained from the cytoplasm.

The preparation of the proteins from a batch of spinach leaves picked June 18, 1924, will illustrate the methods employed. The leaves were removed from the stems within an hour of picking. After washing with water to remove surface soil the wet leaves weighed 22,680 gm. They were plasmolyzed with ether (about 10 pounds were required), enveloped in thick filter cloth, and slowly subjected to a pressure of 2,000 pounds per square inch. The resulting extract, together with the liquid which exudes from the surface of the leaves during plasmolysis, was filtered through pulp, which removed a small amount of soil material that had been washed away from the leaf surfaces. The combined extract measured 20,700 cc., and on analysis was found to contain 378.6 gm. of solids and 12.42 gm. of nitrogen. The residue was allowed to imbibe distilled water and repressed four times successively, the extracts being filtered through the same pulp as that mentioned above. The combined volume was 16,500 cc., and the solution contained 79.7 gm. of solids and 3.88 gm. of nitrogen. The residues from the press contained 982.0 gm. of solids and 63.07 gm. of nitrogen, so that the fresh leaves contained 1,440.3 gm. of solids and 79.37 gm. of nitrogen.

Preparation and Properties of Protein from the Vacuole Fluid.— The first filtered extract mentioned above was heated to 85°C. by passing in steam. A small coagulum formed which was washed successively with water, then with graded strengths of alcohol, and finally with ether. It weighed 10.703 gm.; contained 1.402 gm. of nitrogen and 0.694 gm. of ash, or N, ash-free = 14.02 per cent. This amount of protein represents only 0.74 per cent of the

total leaf solids and 1.76 per cent of the total nitrogen in the leaf. The second filtered extract, made up of the four washings of the press residues, became cloudy when heated to 85°C., but no coagulum settled overnight, showing that the washing did not effect solution of any appreciable amount of protein not originally dissolved in the vacuole juice.

A reduced yield of this vacuole protein is obtained by acidifying instead of heating the vacuole extract. A small flocculent precipitate settles during the course of a few hours, which is soluble in dilute alkali, but, unlike the cytoplasmic protein to be described later, is insoluble in either dilute or strong acid. The distribution of nitrogen as shown in Table I also emphasizes the difference between these two proteins.

TABLE I.

Showing the Distribution of Nitrogen in the Cytoplasmic and Vacuole Proteins from Spinach Leaves.

After hydrolysis with 20 per cent HCl for 16 hours.

	Cytoplasmic protein.		Vacuole protein.	
	Nitrogen.	Protein.	Nitrogen.	Protein.
	per cent	*per cent*	*per cent*	*per cent*
Amide N	6.94	1.14	7.91	1.11
Humin "	2.06	0.33	2.56	0.36
Basic "	26.59	4.32	22.06	3.11
Other " (difference)	64.41	10.46	67.47	9.44
Total N	100.00	16.25	100.00	14.02

Preparation of the Protein from the Cytoplasm.—The pressed residues were passed three times through the meat chopper, using successively finer plates, mixed with 14 liters of distilled water, and passed three times through the Nixtamal mill. The resulting green pulp was enclosed in a bag made of very fine textured silk and the liquid expressed with the hands. The cell residue was removed from the bag, mixed with 14 liters of water, and the whole operation repeated. The volume of the two combined extracts—both heavily charged with green colloidal material —was 25 liters. One-half of this extract was flocculated by means of acid; the chemical examination of this precipitate will form the basis of a subsequent paper. The other half of the extract

was filtered by suction through a pad of paper pulp. This removed all the green opaque colloidal material visible under the microscope, the filtrate being a clear yellow-brown liquid showing a faint green tinge by reflected light. The protein could be flocculated by the addition of a small amount of acid and was soluble in small excess to give a clear yellow-brown solution. 12 cc. of 12 per cent HCl were added and the precipitated protein was allowed to settle overnight. The supernatant liquid was then syphoned off and the fairly dense precipitate of protein—in a volume of about 2,800 cc.—redissolved by adding with constant stirring 40 cc. of 2.5 N NaOH. This solution was slightly opaque and could not be clarified by prolonged centrifuging. It became clear on filtration through paper pulp and the addition of the requisite amount of HCl to the filtrate caused the protein to separate sharply at its isoelectric point. Thus purified the protein was completely soluble, giving a clear solution, in slight excess of either acid or alkali. The precipitated protein was centrifuged off, washed with graded strengths of alcohol, then with ether, and dried in the air. The weight, moisture-free, was 35 gm.; equivalent to 70 gm. for the total extract obtained after grinding. The alcoholic washings were pale green, due to a small amount of chlorophyll. About one-third of the total washings was lost, but allowing for this the weight of alcohol-soluble material did not exceed 2 per cent of the weight of the protein. The ether extract on evaporation gave a negligible amount of a yellow grease.

Analysis of the Cytoplasmic Protein.—The moisture-free protein contains 15.97 per cent of nitrogen; 1.72 per cent of ash; 1.19 per cent of sulfur; and 0.13 per cent of phosphorus. The nitrogen, ash-free, is 16.25 per cent. The yield of 70 gm. of protein, therefore, represents 4.87 per cent of the leaf solids and 14.1 per cent of the leaf nitrogen. Molisch's and Tollen's tests were negative, showing the complete absence of carbohydrate in the preparation. Table II gives the distribution of nitrogen by Van Slyke's method after hydrolysis for 30 hours with 20 per cent HCl.

Properties of the Cytoplasmic Protein.—When flocculated at its isoelectric point the hydrated protein is almost completely insoluble in water and in salt solutions, is coagulated by boiling, and is denatured by strong alcohol to the extent that it is then

soluble in weak alkali only with difficulty, and probably with decomposition. It is freely soluble in a very small excess of alkali to give a clear olive-brown solution and in a very small excess of acid to give a clear but less intensely colored solution. In both cases a pronounced Tyndall effect shows that the solution may be colloidal.

TABLE II.

*Van Slyke Analysis of the Cytoplasmic Protein after Hydrolysis with 20 Per Cent HCl for 30 Hours.**

Total nitrogen in aliquots used for analysis was 0.5886 gm.

	Nitrogen.†	Nitrogen.
	gm.	*per cent*
Amide N	0.0408	6.93
Humin " in acid	0.0045	0.76
" " " lime	0.0084	1.46
" " " amyl alcohol	0.0015	0.25
Cystine	0.0075	1.27
Arginine	0.0812	13.80
Histidine	0.0229	3.89
Lysine	0.0567	9.63
Amino N in filtrate	0.3419	58.09
Non-amino N in filtrate	0.0152	2.58
Total N recovered	0.5806	98.66

Protein.

	per cent		*per cent*
Arginine	6.95	(containing N =	2.24)
Histidine	2.34	(" " =	0.63)
Lysine	8.19	(" " =	1.57)
Total			4.44

* Nitrogen figures corrected for solubility of bases.

† Mean of two determinations.

In weak acid solution the protein is extremely sensitive to the presence of salts; 1 part in 100 of saturated ammonium sulfate or 1 part in 100 of a 10 per cent solution of disodium phosphate is sufficient to cause complete precipitation. It is not precipitated by the addition of an equal volume of 95 per cent alcohol.

In weak alkaline solution the protein is also sensitive to the presence of salts, but less so than in acid solution, for though it is precipitated by the strength of ammonium sulfate mentioned above, it is not precipitated by that of the disodium phosphate. It is slowly precipitated by the addition of an equal volume of alcohol.

Rough colorimetric estimations show that the isoelectric point of the protein lies between pH 4.0 and 4.6. In the plasmolyzed cell the protein must be present as an anion, for when dispersed into colloidal solution by grinding the pressed residues with distilled water the addition of a small quantity of acid is required to flocculate it at the isoelectric point. Since the pH of the vacuole fluid extracted immediately after plasmolysis is also within the range 4.0 to 4.6 it follows that the pH of the protein within the plasmolyzed cell can only be slightly higher than that of its isoelectric point.

The mode of preparation, as outlined above, separates the protein dissolved in the vacuole so readily from that at present under discussion as to leave but little doubt that the latter was present in the plasmolyzed cell as a gel, forming part of the cytoplasm; a deduction which receives support from observations made by following the whole operation with the aid of a microscope. In the living cell the protoplasm usually presents the characters of a liquid; so it is possible that the more rigid gel structure observed after plasmolysis is due to postmortem changes.

The protein isolated by this method represents only one-fifth of the total protein assumed to be present in the cytoplasm; an investigation of the remaining four-fifths is being conducted.

It is proposed to name this new protein "spinacin."

SUMMARY.

By applying the ether method described in a recent paper a new protein, "spinacin," has been prepared from the cytoplasm of spinach leaves (*Spinacia oleracea*). It is insoluble in water and salt solutions, but is soluble in a very small excess of either acid or alkali. It contains 16.25 per cent of nitrogen and is free from carbohydrate.

A NOTE ON DAKIN'S METHOD AS APPLIED TO EDESTIN.*

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Dakin's method[1] for extracting with butyl alcohol the monoamino acids from the products of protein hydrolysis presents many features which supplement the ester method of Fischer.

As he first described the extraction it was conducted in the Steudel apparatus at atmospheric pressure whereby the amino acids were long heated with the boiling alcohol. To this latter fact Dakin attributed the difficulty which he encountered in purifying proline which is extracted from the aqueous solution and is retained dissolved in the butyl alcohol. To avoid this objection Dakin[2] later conducted the extraction *in vacuo*, which reduced the temperature of the boiling butyl alcohol, but did not reduce the time of the extraction.

We have found that the same result can be effected and the method applied on a large scale if the solution of the amino acids is dropped at room temperature into a tall jar containing much butyl alcohol, which is simultaneously rapidly stirred. By repeating this process the monoamino acids are extracted almost completely and can be recovered from their solution in the butyl alcohol by distilling *in vacuo* and washing the residue with ethyl alcohol. By distilling separate portions of the butyl alcohol extract no part of the amino acids need be heated with the alcohol

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Dakin, H. D., *Biochem. J.*, 1918, xii, 290.

[2] Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.

for more than a relatively short time and the temperature need be no higher than that prevailing in Dakin's low pressure method.

We have applied this procedure to the products of hydrolysis of 1,000 gm. of edestin from which the basic amino acids had been previously removed by precipitation with phosphotungstic acid and the less soluble monoamino acids by direct crystallization from water and dilute alcohol.

The filtrate from the last fraction of crystalline amino acids was concentrated to a syrup to remove ethyl alcohol, made up to 2,000 cc. with water, and then allowed to drop rapidly from a separatory funnel into 14 liters of butyl alcohol in a tall jar. While the aqueous solution was falling into the butyl alcohol the latter was violently agitated by a glass stirrer driven by a small motor. After standing a short time the clear butyl alcohol layer was sucked off from the clear aqueous solution and, in portions of about 1,500 cc. each, evaporated to dryness *in vacuo*. The residues were transferred to a Buchner funnel, washed with butyl alcohol, and then with ethyl alcohol, the latter washings being collected separately.

8 liters of the butyl alcohol, thus recovered, were used for a second extraction, as above described, and this process was repeated. In this way 53.0, 21.5, and 14.3 gm. of solid amino acids were successively obtained, or 89 gm. in all, after 223 gm. already had been removed by direct crystallization.

The Aqueous Solution.

An examination of the aqueous solution showed no evidence of the presence of leucine, valine, or phenylalanine. This solution contained 25 gm. of nitrogen, of which 21.8 gm. were amino nitrogen and 3.2 gm. non-amino nitrogen, equal to 3.2 per cent of oxyproline in the dry, ash-free edestin. This amino acid, however, has not yet been chemically identified among the constituents of this solution.

The dibasic acids were removed by precipitation as calcium salts according to Foreman.[3] After removing about 20 gm. of glutaminic acid as hydrochloride the precipitation with calcium was repeated. From the solution of the relatively large precip-

[3] Foreman, F. W., *Biochem. J.*, 1914, viii, 463.

itate about 3 gm. of aspartic acid were removed by boiling with excess of lead hydroxide. It thus appears that most of the glutaminic and aspartic acids had been removed by direct crystallization before the extraction of the remaining monoamino acids by butyl alcohol.

By then applying Dakin's method for the separation of oxyglutaminic acid a not inconsiderable precipitate of a silver salt was produced which, when decomposed and freed from reagents, yielded a syrup from which no crystals could be made to separate.

Although the conditions under which this fraction had been obtained corresponded exactly with those described by Dakin, it gave none of the color reactions characteristic of oxyglutaminic acid. From the failure of this careful search for this amino acid among the products of hydrolysis of so large a quantity of edestin we feel justified in definitely concluding that *edestin does not yield any oxyglutaminic acid.*

When the syrup was dissolved in 100 cc. of water analysis of aliquots showed it to contain 2.5 gm. of solids, containing 16.0 per cent of nitrogen. This high nitrogen content, which excludes any of the known dibasic amino acids, indicates glycocoll. Nevertheless, attempts to obtain glycocoll ester hydrochloride failed.

Since Osborne and Mendel[4] have found that young rats grow well when edestin is their sole source of protein and a few milligrams of a protein-free fraction from yeast furnishes the water-soluble vitamine, the conclusion is justified that *oxyglutaminic acid is not essential for adequate nutrition.*

The Alcohol Solution.

On long standing the ethyl alcohol extract of the residues left by evaporating the butyl alcohol extracts to dryness deposited a small amount of substance on the walls of the flask. From this the clear red-brown alcoholic solution was decanted and distilled *in vacuo* until all the alcohol was removed.

[4] Although earlier experiments (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149) indicated that satisfactory growth could not be obtained with edestin, later unpublished experiments show that this is not the case.

The residue was treated with water and a not inconsiderable amount of a sticky, insoluble product removed by centrifuging and washing with water. Dried over H_2SO_4 this weighed 2.3 gm., contained 9.46 per cent of nitrogen, and was practically all soluble in cold absolute alcohol, ethyl acetate, or chloroform. It was not noticeably soluble in hot dilute hydrochloric acid, but dissolved slowly in boiling dilute sodium hydroxide solution. This behavior indicated diketopiperazines.

Extraction with ether removed a not inconsiderable quantity of a dark brown substance. The residue when crystallized from a little hot absolute alcohol yielded a small quantity of long colorless needles, similar in appearance to crystals subsequently obtained from the proline solution, but the greater part consisted of substances much more readily soluble in alcohol.

The aqueous solution, from which this insoluble product had been removed, contained 44.4 gm. of solids and 4.82 gm. of nitrogen, of which 1.85 gm. were amino nitrogen. The remainder was evaporated until water was removed. The transparent residue was readily soluble in absolute alcohol and no insoluble amino acids separated on standing.

Since so large a part of the total nitrogen of this proline fraction was amino nitrogen the complete solubility of the residue in absolute alcohol was surprising. The reason for this was found in the presence of a notable amount of HCl in the solution. This was derived from the original edestin which was prepared by recrystallization from sodium chloride solution, a method shown to yield hydrochlorides of this protein.

This observation shows how important it is, in using Dakin's method, either as he has directed, or as modified by us, that consideration be given to the presence of chlorides either of the protein or of inorganic bases. Unless chloride ions are removed hydrochlorides of the amino acids, which are readily soluble in butyl alcohol, will pass into the proline fraction.

The clear alcoholic solution, when evaporated *in vacuo* until alcohol was removed, was completely soluble in water, but when this solution was concentrated *in vacuo* a separation of solids occurred which, when dried over H_2SO_4, weighed 0.85 gm. When crystallized from absolute alcohol this substance separated in long colorless needles.

When the filtrate from this substance was evaporated to a clear transparent solid and again treated with absolute alcohol and water, as above described, another separation occurred which, when dried over H_2SO_4, weighed 0.40 gm. and yielded needles on crystallizing from absolute alcohol.

On uniting these fractions and recrystallizing from hot alcohol long, hair-like needles separated which resembled in form and solubility a product similarly obtained from casein. Both melted at 310°C. (corrected), and subsequently decomposed. When mixed the melting point was unchanged. The preparation from edestin contained 12.87 per cent of nitrogen, that from casein 12.90 per cent.

This substance is very sparingly soluble in water, cold ethyl or butyl alcohol, or chloroform. On warming it dissolves and separates, on cooling, in needles. In ethyl acetate or methyl salicylate it is somewhat soluble in the cold, and readily soluble on heating. On cooling it separates in needles. In warm 50 per cent H_2SO_4 it is readily soluble, as it also is in 30 per cent HNO_3. From the latter it separates on cooling in needles, apparently unchanged. It is little, if at all, soluble on heating for a short time with either 5/7 normal HCl or NaOH.

There can be little doubt from the above properties that this substance is a diketopiperazine. Owing to the small amount of pure substance available no evidence of the amino acids which it yields on hydrolysis was obtained further than it gives no reaction for tyrosine, phenylalanine, cystine, or tryptophane.

From the above observations it is evident that, even under the conditions which we employed in applying Dakin's method, the proline fraction is not free from diketopiperazines and consequently Van Slyke's method cannot be used to estimate the proline from the imino nitrogen soluble in alcohol unless the diketopiperazines are hydrolyzed, or the proline esterified and distilled, as was done by Dakin.

Whether these diketopiperazines are normal constituents of the protein molecule, as Abderhalden[5] believes, or are secondary products produced by heating with butyl alcohol, remains to be determined.

[5] Abderhalden, E., *Z. physiol. Chem.*, 1923, cxxviii, 119.

NOTE ON THE BASIC AMINO ACIDS YIELDED BY CASEIN.*

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In determining the basic amino acids in the products of hydrolysis of proteins there is usually a considerable quantity of nitrogen in the filtrate from the lysine picrate which has not been identified as belonging to any known amino acid. Although a part of this doubtless belongs to arginine and histidine, the silver salts of which are not wholly insoluble, nevertheless, the amount of this unidentified nitrogen is generally much more than can thus be accounted for. It is, therefore, a question whether, or not, some other base than the three already known may not be a constituent of proteins.

Having occasion to prepare a large quantity of lysine for nutrition experiments 2 kilos of casein, equal to 1,865 gm., ash- and moisture-free, were hydrolyzed by boiling with a mixture of 1,500 gm. of sulfuric acid and 3,000 gm. of water for 24 hours.

After precipitating the lysine as picrate according to Kossel's well known method, the picric acid was removed from the filtrate and to the concentrated solution 5 per cent of sulfuric acid was added and then phosphotungstic acid reagent as long as a precipitate was produced. After decomposing this precipitate in the usual way 8 gm. of lysine picrate were obtained. In spite of persistent efforts no more lysine picrate could be made to separate from the filtrate. Picric acid was then removed and the solution found to contain 3.14 gm. of nitrogen.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

The filtrate from the phosphotungstate precipitate, last mentioned, contained 2.19 gm. of nitrogen. Since this obviously did not belong to basic substances it needs no further consideration beyond the evidence it affords of the small amount of non-basic amino acids present in the solution from which the lysine picrate was first precipitated by Kossel's method.

Thus only 3.14 gm. of basic nitrogen were present in the solution from which as much as possible of the arginine, histidine, and lysine had been previously separated. This is equal to only 1.1 per cent of the total nitrogen of the casein. Since the silver salts of arginine and histidine are slightly soluble a part of this nitrogen must be ascribed to these amino acids. We may conclude, therefore, that casein contains no significant amount of any hitherto unrecognized base and that the 276.7 gm. of analytically pure lysine picrate which was obtained represent practically all the lysine present in the 1,865 gm. of dry casein. The lysine picrate was equivalent to 107.7 gm. of free lysine, a quantity equal to 5.77 per cent of the casein. This figure confirms those previously obtained in this laboratory.[1]

[1] *Cf.* Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908-09, xxiii, 180.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

VII. THE EFFECT OF CARBON MONOXIDE ON THE ACIDITY OF HEMOGLOBIN.

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(Received for publication, June 12, 1924.)

In the preceding paper of this series (1) it has been demonstrated by the CO_2 titration method that, at reactions between pH 6.8 and 8.0, the increase in the base-binding power of hemoglobin which results when hemoglobin changes from the reduced to the oxygenated state follows a curve calculated on the basis of the assumption, first advanced by Henderson (2) that 1 acid hydrogen in the molecule dissociates more strongly in the oxygenated than in the reduced hemoglobin. This hypothesis and the value of about 29 determined for the ratio $\frac{K'_O}{K'_R}$, of the acid dissociation constants of the one labile group in the oxygenated and reduced forms, respectively, lead to the prediction that the measurable effect of oxygenation on the base-binding power of horse hemoglobin must extend over a pH range as great as from 6.0 to 9.0 (see Fig. 6). The 6.8 to 8.0 range covered in the previous paper (1) is as wide as is practicable for the CO_2 titration method. In order to cover experimentally the wider range theoretically predicted, it becomes necessary to return to the electrometric titration, which, though less accurate than the CO_2 titration, is also less limited in its range.

Oxyhemoglobin itself could not be used because of the effect of the oxygen on the hydrogen electrodes. For this reason we have compared reduced hemoglobin with carbon monoxide hemoglobin in place of oxyhemoglobin. We show in this paper that, within

the pH limits of the CO_2 titration method, carbon monoxide hemoglobin has the same base-binding curve as oxyhemoglobin. There is consequently a considerable basis, though not complete proof, for the assumption that HbCO and HbO_2 bind the same amounts of base at other pH points.

EXPERIMENTAL TECHNIQUE.

The solutions of hemoglobin were prepared by dissolving crystalline oxyhemoglobin (3) in sufficient sodium hydroxide to bring the final concentration to approximately 30 millimolar Na. The final hemoglobin concentrations were approximately 7 millimolar.

In the experiments by the CO_2 titration method the technique used was that described in previous papers of this series (1, 4, 5).

The electrometric titrations of reduced hemoglobin were carried out in the rotating electrode previously described (6) at 20 ± 0.5°; the hemoglobin solution, previously reduced by saturating repeatedly with H_2 at 38°, was then admitted to the electrode vessel. Rotation was continued at a constant rate throughout the experiment. Hydrogen at the rate of about one bubble a second passed through the vessel.

After the voltage had reached and maintained a constant value for 15 minutes the titration was begun. 0.1 N HCl was admitted from a micro burette in 0.06 to 0.10 cc. quantities until the solution had been brought to approximately pH 6. Although it was found that stirring for 10 to 12 minutes between successive additions was usually sufficient for the voltage to reach a constant value, 15 minutes was the time allowed to elapse between readings. In some instances where equilibrium had not been reached in 15 minutes the stirring was continued until a constant potential was obtained.

The technique employed in the titration of the carbon monoxide hemoglobin was comparable in every way with the titration of the reduced hemoglobin except that the hemoglobin was first converted completely to carbon monoxide hemoglobin and the hydrogen pressure was reduced 10 mm. by added carbon monoxide.

The total hemoglobin concentration of each preparation was estimated by Kjeldahl nitrogen determinations, and the concentration of active hemoglobin determined by oxygen or carbon

monoxide capacity analyses of the solutions on the Van Slyke (7) constant volume gas apparatus.

A minor modification in the titrating vessel has been made since it was originally described. Contact between the solution to be titrated and the KCl reservoir was originally made by leaving an ungreased band in the center of the stop-cock at the base of the electrode vessel. This resulted in a diminished sensitivity of the system which was sometimes as great as 1 millivolt. To increase the sensitivity the following substitution has been made. A hole slightly less than 1 mm. has been bored in the stop-cock at right angles to the tail bore and the hole was plugged with washed cotton string. Measurements made with this arrangement are consistently sensitive to 0.2 to 0.4 millivolt. It was found, by comparing the ϵ of our saturated calomel cells obtained with standard phosphate solutions and with 0.1 N HCl, that the former were 7 millivolts lower than the latter and agreed with the usually accepted values for the saturated calomel cell at 20°. This 7 millivolt discrepancy we interpret as representing the difference in diffusion potentials existing when standard phosphate solutions and 0.1 N HCl, respectively, are in the electrode vessel. Since the ϵ of our calomel cells was determined at the beginning of each experiment with 0.1 N HCl, assuming for its pH, 1.08, we have subtracted 7 millivolts from the values thus determined. The ϵ used in each experiment is given at the top of each table.

Calculations of pH and Base Bound by Hemoglobin.

The pH values were calculated from the formula:

$$\text{pH} = \frac{E - \epsilon}{0.0581}$$

where E = potential of the system at 20° corrected to one atmosphere dry H_2; ϵ = value of the saturated calomel cell. The base bound by the hemoglobin was calculated by assuming that all the added base was combined with the hemoglobin and that the added HCl reacted with sodium hemoglobinate to form the acid hemoglobin molecule and NaCl. Specifically, therefore, the sodium hemoglobinate, designated as BHb or BHbCO, was considered equal to the total initial concentration of base minus the HCl added. Since the titrations were continued below the isoelectric point of the hemoglobin the HCl in excess of that needed to neutralize the NaOH originally present is referred to in the tables as HbCl and HbCOCl.

In the experiments by the CO_2 titration method the base bound by hemoglobin was calculated, as in previous papers of this series (1, 4), by subtracting the $BHCO_3$ from the total base added. The pH was calculated by Hasselbalch's equation, the CO_2 solubility being assumed to be proportional to the water content of the solutions, as in the previous paper (1).

RESULTS.

Effect of CO on H_2 Electrode Potentials.

To determine whether the presence of a small amount of carbon monoxide affected the potential of the hydrogen electrode, standard phosphate solutions were placed in the electrode vessel and

TABLE I.

A Comparison of H_2 Electrode Potentials in the Presence and Absence of CO.

pH of solution used.	E.M.F. of cell.	
	H_2	H_2 + 10 mm. CO.
7.50	682.0	682.3
7.75	696.3	695.7
7.95	706.6	705.9

the electromotive force was measured in pure hydrogen and in hydrogen diluted with 10 mm. of CO.

It is evident from the results given in Table I that the presence of carbon monoxide, at least in such a small amount, does not vitiate the results obtained by the hydrogen electrode. We have also compared the potential developed when 0.1 N HCl was in the electrode vessel in the presence and absence of carbon monoxide, without finding any change. It is apparently possible, therefore, to dilute the hydrogen of the hydrogen electrode at 20° with 10 mm. of CO over a wide pH range without influencing the potential developed except in so far as it is affected by the reduction of the hydrogen pressure.

Comparison of the Base Bound by Oxygenated and Carbon Monoxide Hemoglobin by the CO_2 Saturation Method.

The protocols of two experiments are given in Tables II and III. In each experiment the base bound per mol of oxygenated and

TABLE II.

Preparation H −62.

Total [Hb] = 8.38 mM per liter
" [HbCO] = 7.33 " " "
" [Na] = 30 " " "

No.	p_{O_2}	$[HbO_2]$	p_{CO_2}	$[H_2CO_3]$	Total $[CO_2]$.	$[BHCO_3]$	[BHb]	$\frac{[BHbO_2]}{[HbO_2]}$	pH
	mm.	*m*M	*mm.*	*m*M	*m*M	*m*M	*m*M	*m*M	
1	609	6.53	97.3	2.820	24.45	21.63	8.37	1.10	7.055
2	644	6.81	62.3	1.805	20.65	18.84	11.16	1.42	7.188
3	666	6.64	40.3	1.167	17.29	16.12	13.88	1.75	7.310
4	684	6.52	22.1	0.641	13.37	12.73	17.27	2.18	7.468
	p_{CO}	[HbCO]						$\frac{[BHbCO]}{[HbCO]}$	
	mm.	*m*M						*m*M	
5	7	6.97	99.4	2.878	24.32	21.44	8.56	1.09	7.043
6	7	7.22	61.2	1.773	20.12	18.35	11.65	1.45	7.185
7	7	6.87	39.0	1.130	16.65	15.52	14.48	1.81	7.308
8	7	6.69	22.2	0.644	13.07	12.43	17.57	2.19	7.455

TABLE III.

Preparation H−63.

Total [Hb] = 7.30 mM per liter.
" [HbCO] = 7.10 " " "
" [Na] = 30 " " "

No.	p_{O_2}	$[HbO_2]$	p_{CO_2}	$[H_2CO_3]$	Total $[CO_2]$.	$[BHCO_3]$	[BHb]	$\frac{[BHbO_2]}{[HbO_2]}$	pH
	mm.	*m*M	*mm.*	*m*M	*m*M	*m*M	*m*M	*m*M	
1	634	6.43	93.5	2.720	24.85	22.12	7.88	1.15	7.08
2	669	6.35	58.8	1.711	21.05	19.33	10.67	1.54	7.223
3	687	6.23	41.0	1.193	18.44	17.24	12.76	1.84	7.330
4	701	6.32	27.0	0.786	15.68	14.89	15.11	2.16	7.447
	p_{CO}	[HbCO]						$\frac{[BHbCO]}{[HbCO]}$	
	mm.	*m*M						*m*M	
5	7	7.03	100.7	2.930	25.40	22.46	7.54	1.04	7.054
6	7	6.86	60.8	1.769	20.96	19.19	10.81	1.50	7.205
7	7	7.09	38.3	1.114	17.71	16.59	13.41	1.84	7.342
8	7	7.02	22.1	0.644	14.28	13.64	16.36	2.25	7.496

carbon monoxide hemoglobin was determined at 4 different pH values. These results are shown graphically in Fig. 1 where it may be seen that within the limits of our experimental conditions the base bound per mol of oxyhemoglobin is identical with the base bound per mol of carbon monoxide hemoglobin.

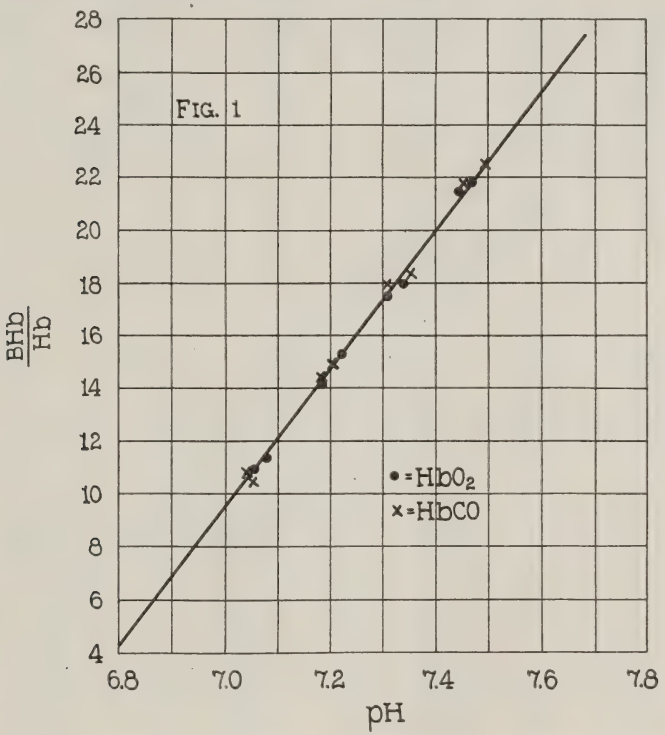

FIG. 1. A comparison of the base bound per mol of hemoglobin when it is combined with oxygen and with carbon monoxide.

A note at the end of Parson's paper (8) on the reaction of the blood calls attention to the fact that the same electrometric pH is obtained whether the blood is saturated with oxygen or carbon monoxide.

TABLE IV.

Preparation H −63.

Total [Hb] = 7.82 mM per liter.
" [HbO_2] = 6.57 " " "
" [HbCO] = 6.57 " " "
" [Na] = 29.3 " " "

Electrometric titration as Hb. ϵ = 248 mv.			Electrometric titration as HbCO. ϵ = 245 mv.		
pH	[HCl]	[BHb]	pH	[HCl]	[BHb]
	mM	mM		mM	mM
8.78	0.0	29.3	8.64	0.0	29.3
8.60	2.2	27.1	8.24	4.5	24.8
8.46	4.4	24.9	7.96	8.8	20.5
8.20	8.4	20.9	7.76	12.4	16.9
8.00	12.3	17.0	7.50	16.9	12.4
7.83	15.6	13.7	7.27	21.4	7.9
7.64	18.9	10.4	7.04	20.2	3.1
7.47	21.6	7.7			
7.26	26.1	3.2			
		[HbCl]			[HbCl]
		mM			mM
7.02	29.8	0.5	6.80	31.4	2.1
6.76	33.9	4.6	6.54	36.4	7.1
6.60	37.1	7.8	6.11	43.7	14.4
6.42	40.1	10.8			
6.15	44.3	15.0			

TABLE IV *a*.

pH	$\frac{[BHb]}{[Hb]}$	$\frac{[BHbCO]}{[HbCO]}$	$\frac{\Delta[BHb]}{\Delta[COHb]}$
	mM	mM	
8.6	3.45	3.76	0.31
8.4	3.10	3.48	0.38
8.2	2.67	3.21	0.54
8.0	2.18	2.85	0.67
7.8	1.74	2.40	0.66
7.6	1.28	1.95	0.67
7.4	0.79	1.44	0.65
7.2	0.33	0.91	0.58
	$\frac{[HbCl]}{[Hb]}$		
	mM		
7.0	0.13	0.37	0.50
		$\frac{[HbCOCl]}{[HbCO]}$	$-\frac{\Delta[ClHb]}{\Delta[COHb]}$
		mM	
6.8	0.59	0.17	0.42
6.6	1.02	0.69	0.33
6.4	1.41	1.16	0.25
6.2	1.79	1.61	0.18

The Electrometric Titration of Reduced and Carbon Monoxide Hemoglobin.

Using the technique described, electrometric titrations of reduced and carbon monoxide hemoglobin were obtained in four solutions of horse hemoglobin.

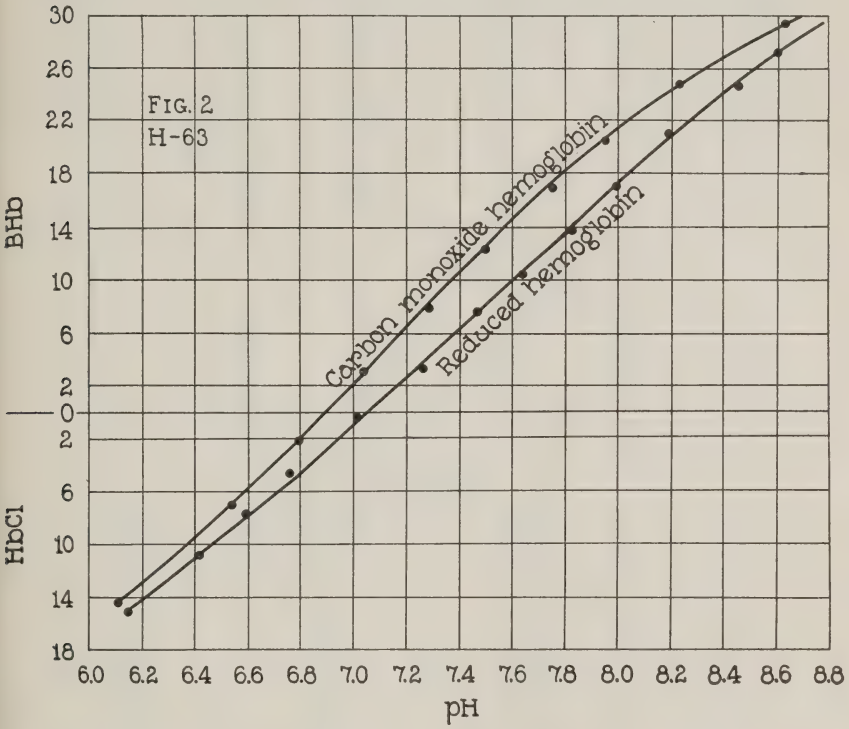

FIG. 2. The electrometric titration curves of reduced and carbon monoxide hemoglobin. Preparation H-63.

The results of these experiments are given in Tables IV to VII. The data are plotted with pH as abscissæ and base bound by hemoglobin as ordinates in Figs. 2 to 5. In each of these experiments and in three other similar ones on dog, rat, and guinea pig hemoglobin, the titration curves of carbon monoxide hemoglobin and reduced hemoglobin, respectively, presented certain characteristic features. In each case the two curves showed a maximum

TABLE V.

Preparation H—64.

Total [Hb] = 7.81 mM per liter.
" [HbO_2] = 7.59 " " "
" [HbCO] = 7.60 " " "
" [Na] = 29.4 " " "

Electrometric titration as Hb. ϵ = 248 mv.			Electrometric titration as HbCO. ϵ = 246 mv.		
pH	[HCl]	[BHb]	pH	[HCl]	[BHb]
	mM	mM		mM	mM
8.45	0.0	29.4	8.25	0.0	29.4
8.26	3.0	26.4	7.92	4.0	25.2
8.05	6.7	22.7	7.72	7.2	22.2
7.85	10.5	18.9	7.55	10.1	19.3
7.66	14.3	15.1	7.41	13.6	15.8
7.48	18.3	11.1	7.28	16.4	13.0
7.32	21.3	8.1	7.01	20.7	8.7
7.18	24.3	5.1	6.98	23.7	5.7
7.03	27.4	2.0	6.84	26.7	2.7
		[HbCl]			[HbCl]
		mM			mM
6.82	31.5	2.1	6.66	31.1	1.7
6.62	34.8	5.4	6.51	33.9	4.5
6.36	38.9	9.5	6.35	37.3	7.9
6.06	42.8	13.4	6.17	40.2	10.8
			5.93	44.0	14.6

TABLE V *a*.

pH	$\frac{[BHb]}{[Hb]}$	$\frac{[BHbCO]}{[HbCO]}$	$\frac{\Delta[BHb]}{\Delta[COHb]}$
	mM	mM	
8.2	3.23	3.70	0.47
8.0	2.79	3.42	0.63
7.8	2.30	3.04	0.74
7.6	1.77	2.58	0.81
7.4	1.22	2.01	0.79
7.2	0.67	1.43	0.76
7.0	0.15	0.79	0.64
	$\frac{[HbCl]}{[Hb]}$		
	mM		
6.8	0.33	0.20	0.53
		$\frac{[HbCOCl]}{[HbCO]}$	$-\frac{\Delta[ClHb]}{\Delta[COHb]}$
		mM	
6.6	0.77	0.37	0.40
6.4	1.15	0.88	0.27
6.2	1.51	1.35	0.16

divergence at about pH 7.4, and approached each other gradually on both sides of this point until at pH 6 and 9 they nearly coincided.

In Tables IV *a* to VII *a* calculations of the base bound per mol of hemoglobin at even 0.2 pH intervals have been made as follows. The base bound per mol of reduced hemoglobin was calculated at

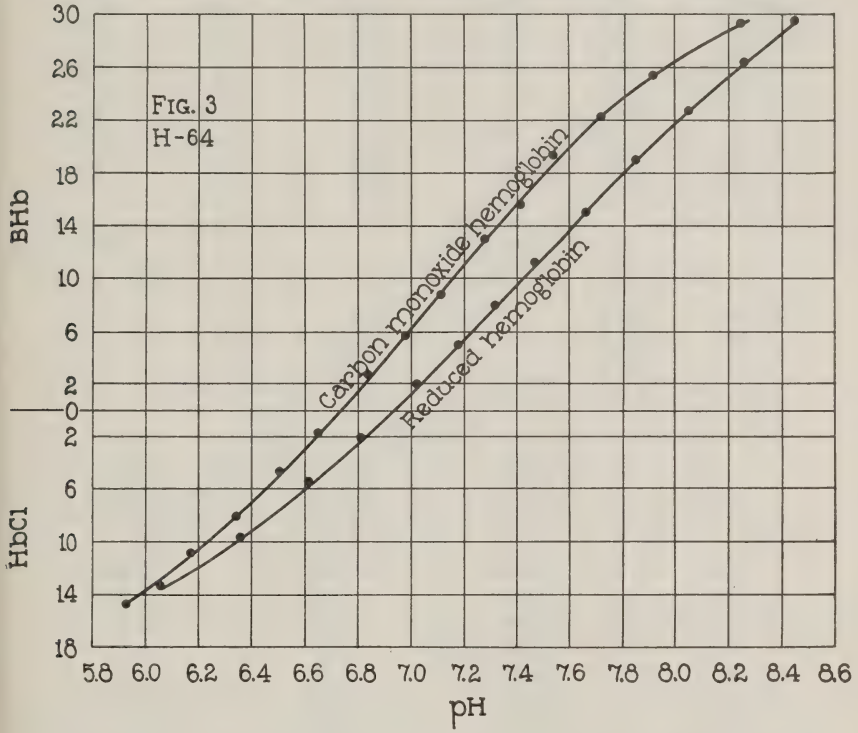

FIG. 3. The electrometric titration curves of reduced and carbon monoxide hemoglobin. Preparation H-64.

0.2 pH intervals by dividing the base bound by the total hemoglobin concentration. To calculate the base bound per mol of carbon monoxide hemoglobin it was necessary first to subtract from the total base bound at each pH the base bound by the inactive and reduced hemoglobin. This subtraction gave the base bound by the carbon monoxide hemoglobin present. The base bound by

TABLE VI.

Preparation H – 66.

Total [Hb] = 7.16 mM per liter.
" [HbO_2] = 6.93 " " "
" [HbCO] = (6.93) " " "
" [Na] = 30 " " "

Electrometric titration as Hb. ϵ = 246 mv.			Electrometric titration as HbCO. ϵ = 246 mv.		
pH	[HCl]	[BHb]	pH	[HCl]	[BHb]
	mM	mM		mM	mM
8.67	0.0	30.0	8.55	0.0	30.0
8.35	4.0	26.0	8.16	4.0	26.0
8.06	8.0	22.0	7.89	7.7	22.3
7.81	12.0	18.0	7.62	11.7	18.3
7.60	16.0	14.0	7.39	15.8	14.2
7.39	19.7	10.3	7.16	19.6	10.4
7.18	23.7	6.3	6.96	23.6	6.4
6.95	27.6	2.4	6.73	27.8	2.2
		[HbCl]			[HbCl]
		mM			mM
6.71	31.6	1.6	6.49	31.7	1.7
6.42	35.5	5.5	6.28	35.8	5.8
6.10	39.6	9.6	6.04	39.9	9.9

TABLE VI *a*.

pH	$\frac{[BHb]}{[Hb]}$	$\frac{[BHbCO]}{[HbCO]}$	$\frac{\Delta[BHb]}{\Delta[COHb]}$
	mM	mM	
8.4	3.71	3.97	0.26
8.2	3.32	3.69	0.37
8.0	2.90	3.33	0.43
7.8	2.43	2.94	0.51
7.6	1.95	2.51	0.56
7.4	1.40	2.03	0.63
7.2	0.89	1.53	0.64
7.0	0.42	1.02	0.60
	$\frac{[HbCl]}{[Hb]}$		
	mM		
6.8	0.03	0.52	0.55
6.6	0.45	0.01	0.46
		$\frac{[HbCOCl]}{[HbCO]}$	$-\frac{\Delta[ClHb]}{\Delta[COHb]}$
		mM	
6.4	0.84	0.49	0.35
6.2	1.17	1.00	0.17

inactive hemoglobin has been found to be identical with that combined with an equivalent amount of reduced hemoglobin from pH 7.0 to 7.6. We have assumed in our calculations that this equality in base-binding power between reduced and inactivated hemoglobin exists also over the pH range covered in our present experiments. The amount of base bound by the carbon monoxide

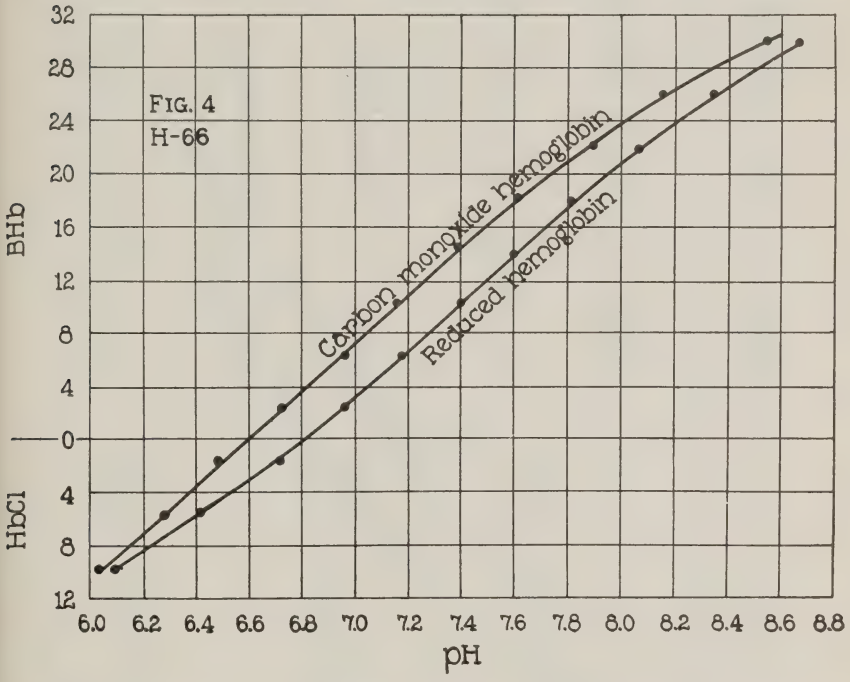

FIG. 4. The electrometric titration curves of reduced and carbon monoxide hemoglobin. Preparation H-66.

hemoglobin divided by the concentration of active hemoglobin in the solution gives us our value for $\frac{[BHb]}{[COHb]}$.

At each 0.2 pH interval the difference between the base bound per mol of carbon monoxide hemoglobin and reduced hemoglobin was calculated and is given in the tables under the heading $\frac{\Delta[BHb]}{\Delta[COHb]}$.

TABLE VII.

Preparation H −69.

Total [Hb] = 6.73 mM per liter.
" [HbO_2] = 6.29 " " "
" [HbCO] = 6.29 " " "
" [Na] = 32.4 " " "

Electrometric titration as Hb. ϵ = 262 mv.			Electrometric titration as HbCO. ϵ = 242 mv.		
pH	[HCl]	[BHb]	pH	[HCl]	[BHb]
	mM	mM		mM	mM
9.54	0.0	32.4			
8.69	6.7	25.7	8.73	5.5	26.9
8.33	10.3	22.1	8.34	8.7	23.7
8.04	14.0	18.4	7.96	12.3	20.1
7.80	17.7	14.7	7.51	17.3	15.1
7.59	21.3	11.1	7.30	21.0	11.4
7.36	25.5	6.9	6.99	25.2	7.2
7.12	29.1	3.3	6.82	28.7	3.7
		[HbCl]			[HbCl]
		mM			mM
6.92	33.0	0.6	6.72	33.0	0.6
6.49	37.3	4.9	6.58	36.4	4.0
6.27	41.0	8.6	6.34	40.3	7.9

TABLE VII *a*.

pH	$\frac{[BHb]}{[Hb]}$	$\frac{[BHbCO]}{[HbCO]}$	$\frac{\Delta[BHb]}{\Delta[COHb]}$
	mM	mM	
8.6	3.69	3.81	0.12
8.4	3.33	3.59	0.26
8.2	2.97	3.39	0.42
8.0	2.52	3.13	0.61
7.8	2.08	2.81	0.73
7.6	1.61	2.43	0.82
7.4	1.10	1.99	0.89
7.2	0.60	1.49	0.89
7.0	0.18	0.90	0.72
	$\frac{[HbCl]}{[Hb]}$		
	mM		
6.8	0.27	0.25	0.52
		$\frac{[HbCOCl]}{[HbCO]}$	$-\frac{\Delta[ClHb]}{\Delta[COHb]}$
		mM	
6.6	0.65	0.49	0.16

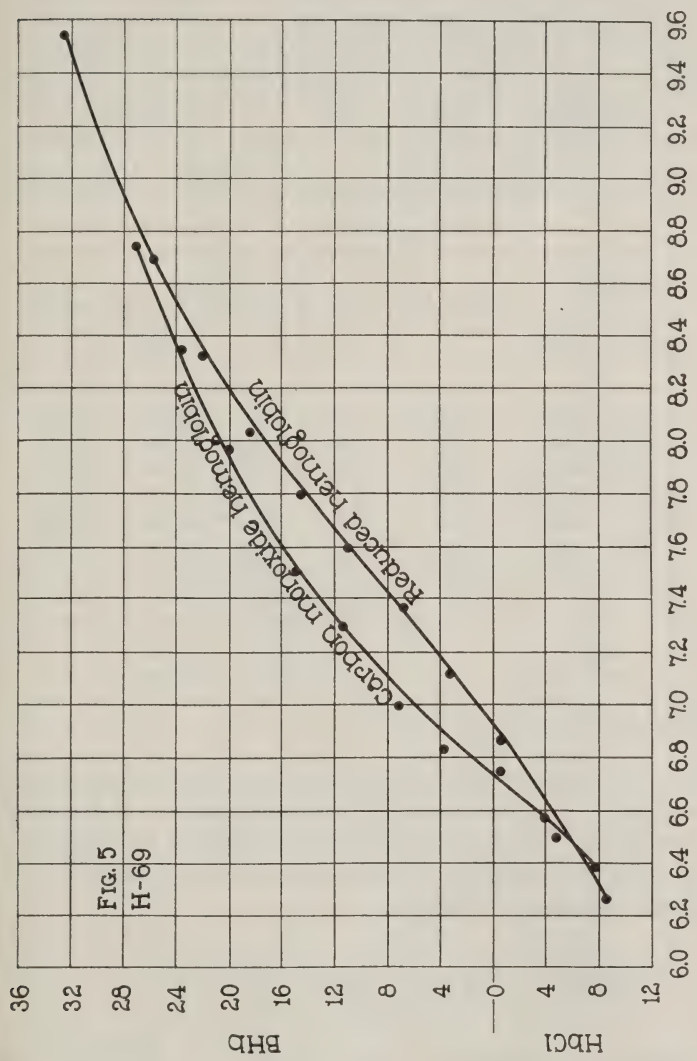

Fig. 5. The electrometric titration curves of reduced and carbon monoxide hemoglobin. Preparation H-69.

Calculation of $pK'_R - pK'_{CO}$.

On the assumption that the dissociation constant of one acid group is increased when reduced hemoglobin is oxygenated, it was shown previously (1) that it is possible either by the solution of simultaneous equations or by graphical methods to calculate from the values of $\frac{\Delta[BHb]}{\Delta[O_2Hb]}$ and pH the change in the dissociation constant of this acid group upon oxygenation of the hemoglobin

TABLE VIII.

pH	$\frac{\Delta[BHb]}{\Delta[COHb]}$ Found.	$\frac{\Delta[BHb]}{\Delta[COHb]}$ Calculated.	$pK'_R - pK'_{CO}$	pH	$\frac{\Delta[BHb]}{\Delta[COHb]}$ Found.	$\frac{\Delta[BHb]}{\Delta[COHb]}$ Calculated.	$pK'_R - pK'_{CO}$
8.6	0.21	0.26	1.28*	7.2	0.72	0.70	1.60
8.4	0.30	0.36	1.36*	7.0	0.62	0.65	1.50
8.2	0.45	0.46	1.52	6.8	0.51	0.56	1.40
8.0	0.58	0.56	1.60	6.6	0.34	0.46	1.20*
7.8	0.67	0.65	1.64	6.4	0.29	0.36	1.28*
7.6	0.72	0.70	1.60	6.2	0.17	0.26	1.16*
7.4	0.74	0.71	1.60	Average $pK'_R - pK'_{CO}$ ... 1.56			

$pK'_R = 8.18$ $\quad K'_R = 6.6 \times 10^{-9}$ $\qquad$ $pK'_{CO} = 6.62$ $\quad K'_{CO} = 2.4 \times 10^{-7}$

* Not included in average.

molecule. By making the same assumption it is possible to calculate similarly the shift in the dissociation constant of the acid group upon the entrance of CO into the hemoglobin molecule.

The expression relating the increase in base bound, the pH, and the labile dissociation constant is:

$$\frac{\Delta[BHb]}{\Delta[COHb]} = \frac{1}{1 + 10^{pK'_{CO} - pH}} - \frac{1}{1 + 10^{pK'_R - pH}} \qquad (1)$$

By substituting successively different pairs of $\frac{\Delta[BHb]}{\Delta[COHb]}$ and pH values, it is possible to calculate the values of pK'_{CO} and pK'_R consistent with our data. The average values for these

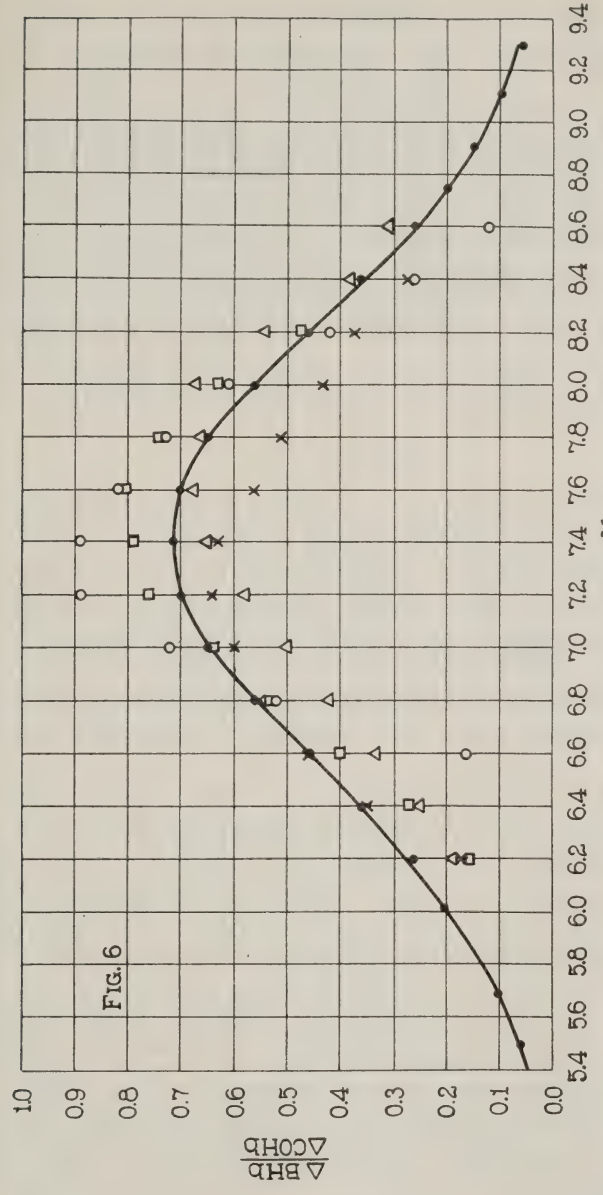

FIG. 6. The solid line is drawn from the formula

$$\frac{\Delta[\mathrm{BHb}]}{\Delta[\mathrm{COHb}]} = \frac{1}{1+10^{6.62-\mathrm{pH}}} - \frac{1}{1+10^{8.18-\mathrm{pH}}}$$

The points indicate particular experiments as follows: Δ, H-63; □, H-64; ×, H-66; ○, H-69.

constants between pH 6.8 and 8.2 were found to be pK'_{CO} = 6.62 and pK'_R = 8.18. The difference, 1.56, indicates a $\frac{K'_{CO}}{K'_R}$ ratio of 36. In Fig. 6 is plotted the curve and the experimental points of Equation 1 in which have been substituted our calculated values for pK'_{CO} and pK'_R. Considering the relative inaccuracy involved in obtaining data on such small amounts of fluid, there is satisfactory consistency between the average of our experimental points and the theoretical curve. Due to the fact that we have at times obtained points which fall both above and below the theoretical curve drawn for the $pK'_R - pK'_{CO}$ difference of 1.56, we at present attach no significance to the deviations from this curve other than that associated with experimental error.

SUMMARY.

The amounts of base bound by solutions of oxygenated hemoglobin and carbon monoxide hemoglobin at 38° have been found by the CO_2 titration method to be identical at the same pH points from pH 7.0 to 7.6. These results, taken with those on oxygenated and reduced hemoglobin previously reported (1) are quantitatively consistent with the assumption that combination of reduced hemoglobin with either CO or O_2 increases to the same extent the dissociation constant of one acid group in the hemoglobin molecule.

In order to test the hypothesis over a wider reaction range, electrometric titration curves at 20° of reduced and carbon monoxide hemoglobin have been obtained from pH 6.2 to 8.6. The quantitative increase in the base bound by hemoglobin upon combination with CO is consistent also throughout this wider pH range with the hypothesis that the dissociation constant of one acid group is increased. At 20° the value estimated from our electrometric data for the dissociation constant of this labile group in the reduced hemoglobin is $K'_R = 10^{-8.18}$. In carbon monoxide hemoglobin, the constant is estimated as $K'_{CO} = 10^{-6.62}$. These values are of the same order of magnitude as the values K'_R and K'_O, respectively, previously obtained (1) by the CO_2 titration method at 38° for reduced and oxygenated hemoglobin.

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CHLORIDES OF SERUM, BLOOD, AND CORPUSCLES IN VARIOUS PATHOLOGICAL CONDITIONS.

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Norgaard and Gram (1), studying the chlorides of the plasma, blood, and corpuscles in anemia and polycythemia, found consistently a fall in blood chloride with rising cell volume and *vice versa*. The concentration of chloride in the cells was approximately only half of that in the serum. The only significant variation in this series was a decrease in cell chlorides in *some* of the cases of pernicious anemia, so that the average cell chloride in this disease was lower than the average normal value (0.31 per cent NaCl).

Later Gram (2) reported a considerable decrease of serum chloride in several diseases, notably in diabetes, pneumonia, uremia, and some cases of nephritis.

Buckman and Edwards (3) reported a consistent decrease in cell chloride concentration with decreasing cell volume, not only in pernicious, but in all cases of anemia and an increase in cell chloride concentration in cases of polycythemia.

In view of these findings it has seemed desirable to study the chloride concentration in serum, cells, and whole blood in cases with (1) low serum chloride and (2) anemias and polycythemias.

Technique.

The blood was taken from an arm vein under oil and defibrinated; then part of it was transferred with precautions against loss

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of CO_2 to a centrifuge tube and whirled.[1] The analysis of blood and serum was made in duplicate by the new Van Slyke (4) method for determining chlorides. Triplicate determinations of cell volume were made with special calibrated hematocrit tubes. The concentration of chloride in the cells was calculated by the formula:

$$C_b = C_s \cdot (100 - V) + C_c \cdot V$$

where

C_b = gm. NaCl per 100 cc. blood.
C_s = " " " 100 " serum.
C_c = " " " 100 " corpuscles.
V = cell volume percentage.

TABLE I.

Six Normal Bloods.

No.	NaCl of serum.	NaCl of blood.	NaCl of corpuscles.	Cell volume.	Calculated blood NaCl.	Relative serum Cl.	Relative blood Cl.	Relative corpuscle Cl.
	gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*per cent*	*gm. per 100 cc.*	*per cent*	*per cent*	*per cent*
1	0.591	0.455	0.314	48.8	0.457	101	100	98
2	0.582	0.457	0.321	47.9	0.460	99	99	100
3	0.594	0.460	0.330	50.7	0.452	101	102	102
4	0.580	0.458	0.336	49.9	0.454	99	101	105
5	0.589	0.451	0.314	50.2	0.453	102	100	98
6	0.587	0.451	0.303	47.9	0.460	100	98	95
Average.	0.587		0.320			100	100	100

When the cell volume is small any errors in the actual analyses are magnified in the calculated cell chloride concentrations.

Since the serum employed in this work differs from that in previous series in that it was separated without loss of CO_2 it was necessary to establish an average normal level for serum chloride. In Table I we have the results in six normal bloods, the average being 0.587 gm. of NaCl per 100 cc. of serum and 0.320 gm. of NaCl per 100 cc. of corpuscles. For these two values it is possible

[1] Technique same as used in obtaining specimens of serum for CO_2 analysis or pH determinations. See Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liv, 121.

to calculate for each cell volume the resultant NaCl concentration of whole blood; this is represented by the heavy line in Fig. 1.

Acting upon the simplest assumption, namely that the cell chlorides vary proportionately with the serum chlorides, it is also possible to draw a series of lines which should represent the approximate chloride concentration of whole blood at different cell volumes for varying serum chloride concentration. This has been done for serum chloride concentrations ranging from 112 to 80 per cent of the average normal value (0.587 per cent). The

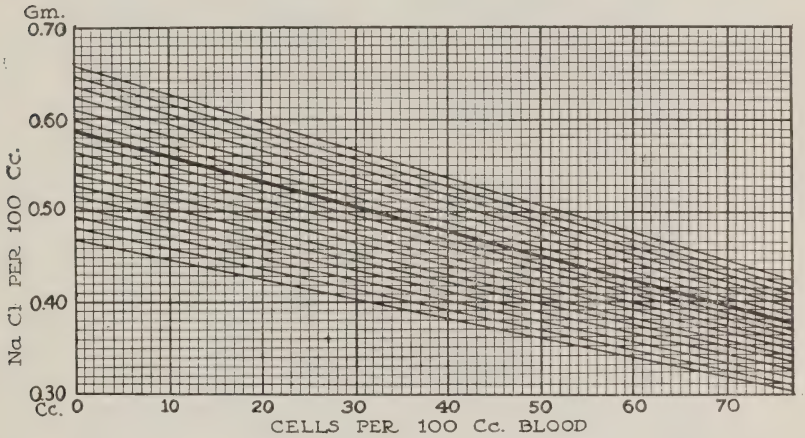

Fig. 1. Relation between blood chloride and cell volume.

interspace between each line represents 2 per cent of the normal, so that one may—when the cell volume is known—at a glance, see how much a whole blood analysis varies from the normal. Beside the values actually found we give in the tables: (1) The blood chloride concentration as it would be according to Fig. 1 on the basis of the observed serum chloride and the observed cell volume ("Calculated blood NaCl"); and (2) the observed chloride concentration of serum, blood, and corpuscles expressed as percentages of the average normal value, the normal value for blood chloride being found by using the heavy line in Fig. 1 ("Relative" Cl values).

TABLE II.

Patients with Normal Serum Chloride.

Date.	Name.	Sex.	Diagnosis.	NaCl of serum.	NaCl of blood.	NaCl of corpuscles.	Cell volume.	Calculated blood NaCl.	Relative serum Cl.	Relative blood Cl.	Relative corpuscle Cl.
				gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*per cent*	*gm. per 100 cc.*	*per cent*	*per cent*	*per cent*
Mar. 26	M.	M.	Polycythemia.	0.580	0.375	0.307	75.1	0.380	99	97	97
Apr. 7	B.	"	Nutritional edema.	0.588	0.461	0.285	42.4	0.471	100	98	89
" 8	W.	"	" "	0.592	0.453	0.280	44.5	0.470	101	97	87
" 14	H.	"	Pernicious anemia.	0.588	0.538	0.298	17.1	0.540	100	99	92

TABLE III.

Cases with Low Serum Chloride.

Date.	Name.	Sex.	Diagnosis.	NaCl of serum.	NaCl of blood.	NaCl of corpuscles.	Cell volume.	Calculated blood NaCl.	Relative serum Cl.	Relative blood Cl.	Relative corpuscle Cl.
				gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*per cent*	*gm. per 100 cc.*	*per cent*	*per cent*	*per cent*
Mar. 13	C.	F.	Diabetes, cancer, hepatitis.	0.511	0.443	0.276	28.9	0.436	87	87	87
Apr. 17	Y.	M.	Uremia.	0.528	0.458	0.246	24.8	0.467	90	88	77
" 21	K.	"	Cardiac edema.	0.552	0.437	0.291	44.0	0.441	94	93	91
May 7	F.	"	Pulmonary tuberculosis.	0.576	0.469	0.313	40.7	0.468	98	98	98

TABLE IV.

*Cases with High Serum Chloride.**

Date.	Name.	Sex.	Diagnosis.	NaCl of serum.	NaCl of blood.	NaCl of corpuscles.	Cell volume.	Calculated blood NaCl.	Relative serum Cl.	Relative blood Cl.	Relative corpuscle Cl.
				gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*per cent*	*gm. per 100 cc.*	*per cent*	*per cent*	*per cent*
Mar. 10	R.	F.	Simple anemia.	0.628	0.564	0.308	20.0	0.581	107	106	96
Feb. 23	K.	M.	Pernicious anemia.	0.641	0.570	0.372	26.0	0.566	109	110	116
Mar. 5	C.	F.	" "	0.610	0.576	0.362	13.7	0.571	104	105	113
Apr. 12	T.	M.	Nephritis.	0.620	0.484	0.315	44.6	0.495	106	103	99
" 24	G.	"	Simple anemia.	0.596	0.519	0.327	28.6	0.520	101	102	102
" 29	M.	"	Polycythemia.	0.603	0.398	0.326	73.9	0.400	103	102	102

* That is, higher than our present series of six normals.

Table II gives the patients with a serum NaCl within the range of our six normal cases. Though the relative corpuscle NaCl is in some instances considerably lower than the relative serum NaCl, yet the relative blood chloride is within 4 per cent of the latter.

Table III contains the cases with a marked reduction of the serum chloride. Here also the relative corpuscle NaCl is in some

TABLE V.

Relation of Corpuscle Chloride to Cell Volume.

Cell volume.	Relative corpuscle Cl.	Corpuscle chloride index.
per cent	*per cent*	
75.1	97	98
73.9	102	99
50.7	102	101
50.2	98	96
49.9	105	106
48.8	98	98
47.9	100	101
47.9	95	95
44.6	99	93
44.5	87	86
44.0	91	97
42.4	89	89
40.7	98	100
28.9	87	100
28.6	102	101
26.0	116	106
24.8	77	86
20.0	96	90
17.1	92	92
13.7	113	109

instances markedly lower than the relative serum NaCl; the relative blood NaCl, however, agrees within 2 per cent with the latter.

We have in Table IV cases with serum chloride higher than any of our six normal cases. The relative corpuscle NaCl in several cases varies markedly in both directions from the relative serum chloride, while the relative blood chloride agrees with the latter to within 3 per cent.

Turning to the second problem, the behavior of the corpuscle chlorides in polycythemia and anemia, all the cases are arranged according to cell volume in Table V. The two other columns of

this table give: first, the corpuscle chloride in percentages of the average normal ("Relative corpuscle Cl") and, secondly, a "Corpuscle chloride index" obtained by multiplying the ratio

$$\frac{\text{Relative corpuscle NaCl}}{\text{Relative serum NaCl}}$$

with 100. While fairly large variations in both relative corpuscle chloride and corpuscle chloride index occur, there is no evidence that decreased cell volume is accompanied regularly by a decrease in corpuscle chloride or *vice versa*.

SUMMARY.

1. The chloride concentration of serum, blood, and corpuscles in various conditions has been studied by the new Van Slyke method of chloride analysis.

2. The increase and decrease in serum chloride in various diseases is accompanied by a corresponding percentage change in whole blood chloride. A graph is given which allows an estimation within 3 per cent of blood chloride from serum chloride or *vice versa*, when the cell volume is known.

3. Whether or not the variation in chloride concentration of the serum—which is independent of cell volume—is taken into account, there is no evidence that the chloride concentration in the corpuscles rises and falls with the cell volume percentage.

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A NOTE ON THE ESTIMATION OF CARBON DIOXIDE IN SERUM IN THE PRESENCE OF ETHER BY THE VAN SLYKE METHOD.

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(Received for publication, January 21, 1924.)

In the course of investigations in this laboratory upon the effects of ether anesthesia upon the acid-base balance, studies were undertaken and will be reported in a later publication on the effect of the addition of ether to blood *in vitro* upon the CO_2-combining capacity of the serum. The concentration of ether in the blood which is associated with deep anesthesia is, according to both White (1923) and Ronzoni (1923), about 1.0 to 1.8 mg. per cc. Our studies were commenced accordingly with about these concentrations of ether added to dog's blood *in vitro*.

It was found that the presence of ether in serum in such concentration interferes with the analysis for CO_2 by the usual Van Slyke technique with either the volumetric apparatus (Van Slyke, 1917; Van Slyke and Stadie, 1921) or with the manometric apparatus (Van Slyke, 1921). This is due to the fact that a considerable portion of the ether which is extracted from the acid solution and is measured with the mixed gases is, subsequently, during the absorption of the CO_2 with alkali, reabsorbed by the NaOH and is absent from the final measurement. The magnitude of the error involved can be seen from the final column of Table I or from Column G of Table II. The presence of oxygen and nitrogen offer no serious difficulty of this sort since their solubility coefficients at 25°C. are very low, being only about 1/2,000 that of ether, and the amounts passing into solution in the absorbing alkali are negligible. Such a volatile substance as acetone likewise offers no difficulty of this sort since its solubility

coefficient is thirty times greater than that of ether at 25°C. and the amount extracted from the acid solution in the original extraction is negligible. The presence of even a few millimols, however, of a gas with an intermediate solubility coefficient in the same region as that of ether must introduce an error similar to that here described.

The error can be eliminated in the case of ether by a technique to be described using the manometric apparatus of Van Slyke. This consists essentially of reextraction of the alkaline solution after absorption of the CO_2 with alkali. The CO_2 remains held by the alkali, but the ether is again extracted in part into the gas phase. An empirical correction must be introduced owing to a greater solubility of the ether in the acid solution than in the

TABLE I.

Analyses on Volumetric Machine of a Na_2CO_3 Solution with and without Ether, Using Ordinary Technique.

Ether added.	Gas extracted.	t	Barometric reading.	Gas after absorption with N NaOH.	Gas absorbed with NaOH.	Corrected to 0°, 760 mm.
mg. per cc.	*cc.*	*°C.*	*mm.*	*cc.*	*cc.*	*mM*
None.	0.395	22.8	757.7	0.044	0.351	14.90
"	0.400	23.2	757.7	0.050	0.350	14.85
1.1	0.431	23.2	757.7	0.050	0.381	16.15

alkaline solution. It may be noted in passing that the use of saturated KOH for absorbing the CO_2 instead of the usual N NaOH does not repress the absorption of ether sufficiently to make such a modification significantly useful.

With carbonate solutions, repeated extraction of the acidified solution gives practically identical readings. Furthermore, it was found that after absorption with alkali, when the proportions of acid to alkali used are 0.5 cc. of N lactic acid to 1 cc. of N NaOH, extraction of the alkaline solution under negative pressure can be carried out without measurable passing of CO_2 out of the solution. When this is to be done, both the lactic acid and the NaOH used must be previously extracted and kept under oil. The results of analysis of carbonate solution or serum without ether by this method are identical within the error of duplicate readings, with analysis obtained with the usual technique.

When this method of reextracting was applied to water containing ether it was found that more of the ether was extracted from the alkaline solution than from the acid solution. The difference amounted to about 15 per cent increase of the ether in the gas phase, and this in spite of a 20 per cent increase in the volume of the liquid phase. Apparently, therefore, the ether is less soluble in the alkaline than in the acid solution.

When this method of reextracting after absorbing with alkali is applied to carbonate solution or to serum containing ether, if a correction for the increase in amount of ether present in the gas phase, after rendering alkaline, can be introduced, the CO_2 present can be determined. It has been found that with either of two manometric machines tested, this correction can be based with sufficient precision upon the difference between the reading after simple absorption with NaOH at approximately atmospheric pressure, and the reading obtained after reextraction of the alkaline solution. The empirical formula employed is given below.

The technique employed, which is merely an extension of the Van Slyke-Neill technique (1924), is as follows:

1 cc. of water, slightly acidulated with lactic acid, is introduced into the manometric machine with 2 drops of octyl alcohol and twice shaken out. This is run up into the cup and beneath it is introduced 1 cc. of the solution or serum to be analyzed. When the solution and water have run into the neck of the cup, extracted N lactic acid is added and introduced to the 2.5 cc. mark of the machine. After sealing, the Hg is dropped to the 50 cc. mark and the acidulated mixture shaken 3 minutes. The fluid is run up to the 2 cc. mark and the manometer read (see Column A, Table II). The Hg is run down again to the 50 cc. mark, the fluid reshaken for $1\frac{1}{2}$ minutes, and read again at the 2 cc. mark. If the readings vary by more than a few tenths of a millimeter this is repeated further.

The fluid is then run up until there is only a slight negative pressure. There is then run in either 1 cc. of extracted N NaOH or 0.5 cc. of extracted 2 N NaOH. After sealing, the Hg is dropped slowly until the water meniscus is just below the 2 cc. mark; it is then brought to the 2 cc. mark and the manometer read (see Column B, Table II). The Hg is then dropped to the 50 cc. mark, the alkaline mixture shaken 3 minutes and run up to the

TABLE II.

Analyses on Manometric Machine of Carbonate Solutions and of Serum with and without Ether.

Solution.	Ether added.	t	*A* Readings of manometer after repeated extractions.	*B* Manometer after reabsorption with NaOH.	*C* Readings of manometer after repeated extractions.	*D* Average of A.	*E* Average of C.	*F* Δ_a Column B − Column D, corrected for NaOH added.	*G* Apparent $[CO_2]$ from Δ_a (ordinary technique).	*H* Δ_r Column E − Column D, corrected for NaOH added.	*J* Apparent $[CO_2]$ from Δ_r (special technique).	*K* True $[CO_2]$. Column J + empirical correction.
	mg. per cc.	*°C.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mM*	*mm.*	*mM*	*mM*
0.0152 M Na_2CO_3	None.	25.1	765.3	898.0	898.8	765.3	898.8	131.5	15.08	132.3	15.17	15.17
			765.3		898.8							
	"	25.4	766.0	899.1	900.2	766.0	900.1	131.9	15.10	132.9	15.21	15.21
			766.0		900.0							
	1.8	25.8	703.5	894.8	828.9	704.2	829.8	189.4	21.68	124.4	14.23	15.21
			704.5		830.5							
			704.5		829.8							
					830.0							
Serum No. 1.	None.	25.0	666.2	879.1	880.0	666.1	880.0	211.8	24.31	212.7	24.41	24.41
			666.0		880.0							
	"	25.2	666.0	880.0	879.8	666.0	879.8	212.8	24.40	212.6	24.39	24.39
			666.0									
	0.7	25.7	644.0	877.4	857.3	644.4	855.8	231.8	26.51	210.2	24.07	24.39
			645.2		855.2							
			644.0		854.1							
					856.5							

Serum No. 2.	Trace.	24.7	677.4 678.1	891.3	889.7 889.6	677.8	889.7	212.3	24.40	210.7	24.21	24.23
	"	24.8	677.0 677.6	893.0	889.1 889.0	677.3	889.1	214.5	24.65	210.6	24.20	24.26
	1.4	25.1	657.8 658.3	890.4	867.5 867.5 867.5	658.1	867.5	231.1	26.52	208.2	23.90	24.24
	2.8	24.9	646.1 646.2	890.0	856.1 856.7 852.7 851.8 849.5 853.9 852.8 854.6	646.2	853.5	242.6	27.85	206.1	23.69	24.24
Serum No. 3.	None.	22.8	620.1 620.4	807.9	807.1 807.1	620.3	807.1	187.0	21.69	186.2	21.60	21.61
	1.1	24.3	595.0 594.9	806.9	779.8 778.1 776.6 778.8	595.0	778.5	211.3	24.35	182.9	21.08	21.51

2 cc. mark, and the manometer read (see Column C, Table II). The extraction and reading are repeated as often as the variation in reading seems to warrant.

In the presence of ether, the rate of running up the alkaline solution to the 2 cc. mark for measurement introduces more variation than in the absence of ether. The technique employed has been to bring the solution to the 2 cc. mark slowly with an even rise, free from bobbing of the meniscus. It has been found necessary to repeat the extraction and reading oftener the greater the amount of ether, shaking for 3 minutes in the original reextraction and for $1\frac{1}{2}$ minutes for each subsequent reextraction. In the presence of ether the variation in reading on extraction of the alkaline solution is greater than with the acid solution and more readings are accordingly necessary. The individual readings are tabulated in Columns A and C of Table II, the averages, respectively, in Columns D and E.

The calculation including the empirical correction for change in the ether in the gas phase is as follows:

$$[CO_2] = [CO_2]_{app} + \{0.15^* (\Delta_a - \Delta_r)\}$$

The empirical correction in braces is to be applied only when $\Delta_a > \Delta_r$.

Δ_a = difference in mm. of Hg in pressure at constant volume after extraction when acidified and after absorption with extracted NaOH at nearly atmospheric pressure. This is the difference used in calculation in the ordinary technique (see Column F, Table II).

Δ_r = difference in mm. of Hg in pressure at constant volume after extraction when acidified and after reextraction of the alkaline solution (see Column H, Table II).

$[CO_2]_{app}$ = apparent CO_2 concentration calculated from Δ_r using Van Slyke's (1921) formula[1] $[CO_2]_{app} = \frac{i\,a\,\Delta_r}{2.24 \times 760}\left(\frac{273}{T} + \frac{S}{A-S}\alpha_{CO_2}\right)$ in which $[CO_2]_{app}$ is expressed in millimols per liter instead of volumes per cent and Δ_r is introduced in place of $m-n$ of the original formula.

$[CO_2]$ = true CO_2 concentration of millimols per liter.

i = empirical factor for reabsorption of CO_2 while running up the fluid to the 2 cc. mark (for our machines 1.017).

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* The value of this constant must probably be determined for each machine and perhaps for each individual's technique.

[1] In the accompanying paper by Van Slyke and Neill the equivalent of the above formula somewhat simplified is given as Equation 5. (Editor's note.)

a = constant volume at which pressure is measured.
S = volume of liquid in machine during initial extraction of gases.
A = total volume of machine.
$\frac{S}{A-S}\alpha_{CO_2}$ = dissolved CO_2 (not including that absorbed while running up fluid to the 2 cc. mark).

In studies previously published by Van Slyke, Austin, and Cullen (1922) and by Cullen, Austin, Kornblum, and Robinson (1923) on the acid-base equilibrium in ether anesthesia the error introduced into the CO_2 analyses by the presence of ether is in most experiments much smaller than the errors shown in this paper. Many of our studies were made on blood taken after recovery of consciousness, following anesthesia, when the ether concentrations do not exceed from one-fifth to one-tenth those used in this study. The analyses in our first paper were made with the volumetric machine in which the magnitude of the error from ether is somewhat less than in the manometric machine. Furthermore, such differences as exist between the true and reported CO_2 contents in those studies are in the direction of lower true CO_2 contents than the reported values under anesthesia, and since the observations were used as evidence of a fall in alkaline reserve the effect of correcting the CO_2 analyses would be to make the fall in alkaline reserve more marked and would not alter the nature of our conclusions with regard to it. In the first three experiments of Van Slyke, Austin, and Cullen where the CO_2 tension and pH are determined by interpolation of the observed CO_2 content of the serum as drawn upon an experimentally determined CO_2 absorption curve, probably the reported CO_2 tensions under ether are too high and the pH under ether too low. In all our other experiments pH is directly determined colorimetrically. In these other experiments the reported CO_2 tensions under ether calculated from observed apparent CO_2 content and from observed pH are probably too high by possibly as much as 10 per cent. We have based no conclusions, however, upon these calculated CO_2 tensions.

It is evident that the difficulty reported here in the analysis of CO_2 in the presence of ether may occur with other volatile substances that have similar distribution coefficients. A test of the effect of acetone on CO_2 analysis shows that even in a concentration

equal to that of the total ketones expressed as acetone found in severe diabetic acidosis no significant error is introduced into the CO_2 analysis (see Table III). If the solubility coefficient of acetone in water at 25° be calculated from the data of Taylor (1900) it will be found to be thirty times that of ether so that the error due to acetone in the concentration used in these studies should be within the error of the method.

A simple method of detecting the presence of a disturbing volatile gas is the reextraction of the alkaline solution after the reading following absorption with alkali. In the absence of a disturbing volatile gas such reextraction alters the reading by at most 1 mm. and as often decreases as increases the reading. In the presence of a volatile gas reextraction significantly increases the gas above the alkaline solution.

TABLE III.

Analyses on Manometric Apparatus of a Na_2CO_3 Solution with and without Acetone, Using Ordinary Technique.

Solution.	Acetone added.	t	Manometer after extraction.	Manometer after reabsorption with NaOH.	Difference corrected for NaOH added.	$[CO_2]$
	mg. per cc.	*°C.*	*mm.*	*mm.*	*mm.*	*mM*
0.0158 M Na_2CO_3	None.	23.4	761.9	900.0	136.9	15.82
	"	24.9	755.1	893.2	136.9	15.72
	0.8	24.5	755.9	894.5	137.4	15.80

SUMMARY.

1. Attention is called to a difficulty in CO_2 determination by Van Slyke's method when the solution for analysis contains gases, with solubility coefficients in the neighborhood of that of ethyl ether.

2. A modification of Van Slyke's method is described for use in analyzing solutions for CO_2 in the presence of ethyl ether.

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LOW NITROGEN METABOLISM WITH LOW CARBOHYDRATE DIET IN DIABETES.

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Physiologists generally agree that a low N metabolism is best obtained by giving (on low N intake) a high calorie diet, and especially a great amount of carbohydrates. The quantity of N metabolism has generally been expressed by the quotient of protein used in the body, calculated by the amount of N in the urine (in grams), divided by the body weight (in kilos). Sivén has obtained the smallest value for that quotient (namely, 0.22 in a 6 day experiment) so far reported. Hindhede has reported a quotient of 0.24 in an 8 day experiment, and 0.25 for a 28 day experiment. Rabe and Plaut found perhaps still lower values on an individual who fletcherized. These authors have not published the exact figures for the N in the urine. As far as one can judge from the nitrogen curve the value for the N quotient was about 0.19 to 0.20.

Landergren concluded, mainly on theoretical grounds, that a daily intake of 40 to 50 gm. of carbohydrates is necessary to attain the lowest value for the metabolized N. Unless this quantity of carbohydrates is given, Landergren believes that the organism uses its own protein to secure the required amount of sugar. Rubner has verbally expressed to me that 60 gm. of carbohydrates are the minimum quantity required.

I have for years treated grave diabetes by a diet, very low in protein, poor also in carbohydrates (especially no bread or other farinaceous foods), but high in fat. This method was developed for therapeutic purposes, but proved also to be of great interest as

indicating the manner in which we can attain a very low N metabolism. Fr. Müller, using a similar diet in diabetes, has obtained values for the N output in the urine as low as 2.1, and 1.7 gm. per day.

In six different patients I have obtained lower values for the nitrogen quotient than has hitherto been reported, namely 0.14 to 0.21, and these values have been calculated for a much longer observation period as will be seen from Table I.

TABLE I.

Diabetic Patients.

Patient No.	Age.	No. of days on which calculation is based.	Average value per day.				Body weight.	Indicated quotient for amount of metabolized N.
			N in urine.	Intake of:				
				N	Carbohydrate.	Fat.		
	yrs.		*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
124	17	12	1.3	3.25	42.4	196.6	57.0	0.14
124	17	36	2.0	3.9	46.0	215.7	57.0	0.18
166	56	20	1.6	3.7	34.3	150.2	63.8	0.16
119	58	30	3.1	5.6	54.1	131.9	108.1–102.7	0.187
169	61	20	2.7	3.1	55.3	195.55	86.7	0.19
169	61	60	3.0	4.9	71.35	213.1	86.7	0.21
114 b	55	29	2.9	3.0	77.0	222.6	82.8	0.21
240	53	30	3.3	3.1	43.5	149.5	95.3	0.21
15 a	48	28	3.1	5.2	53.6	261.4	88.5–86.0	0.22
113 b	32	26	2.1	2.5	43.0	150.9	59.8–57.5	0.22
157	43	61	2.3	3.2	43.3	138.05	64.8	0.22

94 diabetic patients in our clinic have shown a nitrogen quotient less than 0.40 on daily diets ranging from 1.6 to 6.1 gm. of N, 93 to 350 gm. of fat, and 14 to 100 gm. of carbohydrates. The average figures on the six patients with a carbohydrate intake smaller than 30 gm. are given in Table II.

These observations show that in diabetic persons with a carbohydrate intake much below 40 gm. per day, with liberal amounts of fat, a very low N metabolism may be maintained over long periods. It is true that these are diabetic persons, but it does not seem probable that there is any difference in this phase of metabolism between the normal and the diabetic organism. Our

results indicate *that the sparing influence of fat on the quantity of metabolized N is greater than has been assumed.*

These observations were made on hospital patients and it must be admitted that it has not been possible to control these subjects as accurately as an animal confined to a metabolism cage. Hence, one might perhaps believe that the low urine nitrogen is in part due to loss of urine. We admit the possibility that some of these individuals may have lost a portion of the urine occasionally, but all the patients showed willing and intelligent cooperation in the work. The error from loss of urine is, therefore, too small to

TABLE II.

Diabetic Patients.

Patient No.	Age.	No. of days on which calculation is based.	Average quantity of N in urine.	Average quantity of intake of:			Indicated quotient for metabolized N.
				N	Carbohydrate.	Fat.	
	yrs.		*gm.*	*gm.*	*gm.*	*gm.*	
20	36	40	2.8	2.8	14.5	243.4	0.28
20 b	36	50	3.4	3.45	14.1	219.7	0.37
32	30	30	3.1	3.3	16.5	261.9	0.305
243	24	40	2.8	2.3	25.9	180.0	0.37
96	64	42	3.7	2.75	26.2	113.6*	0.275
244	66	20	4.3	1.9	26.4	189.1	0.35

*This patient began with a body weight of 88.5 kilos; a diminished weight seemed desirable; he finished with 79.7 kilos.

invalidate the results. In estimating the importance of an error due to urine loss, it may be noted that we lay no great stress on the extremely low nitrogen quotients as such. On this point the difference between our data and those of Sivén is only one of degree. The essentially new in our results is the marked ability of fats to maintain a low nitrogen metabolism, a nitrogen-sparing action of fat almost as great as that of carbohydrates, and this conclusion is not invalidated by slight errors from loss of urine.

Additional evidence for the marked sparing action of fats on protein metabolism is submitted in Table III.

In Patient 20 it may be argued that it was the increase of the carbohydrates rather than the fats that had the sparing action on the amount of metabolized N, so that the rise of the N intake from

1.4 to 6.3 gm. caused a rise of the metabolized N only from 2.4 to 3.1 gm.

In Patients 32, 40, and 104 the increase in the intake of fat is so much greater than that of the carbohydrates that the reduced nitrogen metabolism must be ascribed to the former.

In Patient 42 the comparison is to be made between the first and third periods. Here we have a lowering of the carbohydrate intake at the same time as the fat intake was increased. The

TABLE III.

Diabetic Patients.

Patient No.	Age.	Initial blood sugar.	Period.	Average amount of N in urine.	Patient had the following average intake of:		
					N	Carbohydrate.	Fat.
	yrs.	*per cent*		*gm.*	*gm.*	*gm.*	*gm.*
20	36	0.30	Dec. 18–Jan. 16	2.4	1.4	13.9	239.4
			Jan. 17–Feb. 13	3.1	6.3	24.1	297.8
40	47	0.23	" 26– " 14	4.9	3.6	68.85	200.15
			Feb. 15–Mar. 2	5.4	6.65	84.1	327.9
32	30	0.34	Jan. 17–Jan. 31	5.0	1.5	13.1	171.6
			Feb. 1–Feb. 15	3.3	2.3	16.1	252.9
			" 16–Mar. 2	3.3	6.7	24.1	313.4
104	15	0.22	May 5–June 18	5.5	2.8	59.1	143.3
			June 19–Aug. 2	4.3	4.8	62.7	234.6
42	18	0.31	Jan. 28–Feb. 16	4.8	4.9	79.6	252.65
			Feb. 17–Mar. 17	5.7	5.4	74.8	342.0
			Mar. 18–Apr. 26	5.1	6.1	74.3	364.4
103	36	0.27	Apr. 24–June 13	5.3	2.7	50.7	152.5
			June 16–July 29	5.1	4.7	35.1	293.7

sparing influence that was attained for the quantity of metabolized N is, however, not so great. In Patient 103, on the contrary, we had a considerable lowering of the carbohydrate intake parallel with the increase in the fat. The sparing influence on the metabolized N is marked.

Physiologists are, on the whole, agreed that it is possible to attain very low values for the N metabolism, only if the amount of calories is abundant. The calorie value of the diet has, in most of my patients with very low N metabolism, been high.

However, some patients have shown very low N metabolism on low calorie diets. These data are given in Table IV.

We have thus obtained by a combination of low protein, low carbohydrate, high fat diets a lower N metabolism than hitherto reported by physiologists, and this has been secured even on a low calorie intake. We do not believe that this is due to a difference in metabolism between the normal and the diabetic person. The difference in results is more likely due to the fact that the experiments by physiologists (except those of Hindhede) have

TABLE IV.

Diabetic Patients.

Patient No.	No. of days on which the calculation is based.	Average quantity of N in urine.	Average quantity of intake of:			Calorie value.	Body weight.	N quotient.
			N	Carbohydrate.	Fat.			
		gm.	*gm.*	*gm.*	*gm.*		*kg.*	
151	32	2.7	1.6	34.2	112.8	1,230	64.6	0.26
96	28	3.4	3.4	29.9	117.1	1,300	88.5–79.7	0.25
159	20	3.2	4.2	87.3	93.6	1,320	68.1	0.28
199	40	3.5	5.2	39.3	121.55	1,400	68.4	0.32
179	28	3.4	2.4	35.5	130.5	1,420	69.1	0.31
157	61	2.3	3.2	43.3	138.0	1,450	64.8	0.22
119	30	3.1	5.6	54.1	131.9	1,600	108.1–102.7	0.19
166	20	1.6	3.7	34.3	150.2	1,625	63.8	0.16
167	25	2.6	3.9	81.85	123.1	1,625	43.7	0.37
246	30	3.3	3.1	43.5	149.5	1,645	95.3	0.21
144	24	4.3	4.7	47.0	147.7	1,645	78.6	0.34
113 b	26	2.1	2.5	43.0	150.9	1,645	59.8–57.5	0.22

extended over a few days only, while these diabetic patients have used the same diet for long periods, very often for a month or more. Consequently, in them the organism has had more time to adjust itself to the diet.

The amount of the protein intake is obviously of importance as regards a low N metabolism. Sivén, Hindhede, Klercker, Thomas, and Landergren failed to obtain N equilibrium on the quotients below 0.30. N equilibrium or a positive N balance is reported by Klercker, Folin, and Klemperer when the N quotients range from 0.29 to 0.35.

This seems to indicate that the lowest N metabolism is obtained only when the protein intake is so low as to induce a negative N balance. But we see in our patients (Table I), Nos. 119, 124, 166, 169, and 15 a, an N equilibrium or positive balance with N quotients well below 0.30 (0.14 to 0.22)[1] and additional cases of N equilibrium on N quotients below 0.30 are given in Table V.

In the great majority of the patients there has been a decrease in the N output in the urine during the fasting days, even when

TABLE V.

Diabetic Patients.

Patient No.	No. of days on which the calculation is based.	Average quantity of N in urine.	Average quantity of intake of:			Body weight.	N quotient.
			N	Carbohydrate.	Fat.		
		gm.	*gm.*	*gm.*	*gm.*	*kg.*	
247	26	2.7	4.0	78.4	281 8	72.2	0.23
19	40	3.0	4.0	55.7	320.0	73.5–85.0	0.24
108	28	2.55	5.6	108.6	331.5	62.0–65.0	0.25
192	20	3.0	4.5	73.5	219.2	74.9	0.25
109 b	21	2.3	4.0	34.1	201.5	52.0	0.26
176	20	3.3	8.7	66.5	158.1	77.0	0.26
194	20	2.8	4.6	66.8	149.3	64.7	0.26
145	20	2.8	4.8	71.5	287.3	62.4	0.27
248	20	3.4	6.8	80.7	245.8	76.9	0.27
147	50	2.35	3.9	57.1	239.2	51.5	0.28
110 b	54	3.3	5.3	61.6	185.5	73.7	0.285
202	54	3.75	7.55	52.5	257.2	78.7	0.29
127	30	3.8	6.0	59.3	240.4	82.5	0.28
53	60	2.7	5.55	74.5	322.4	51.2–63.2	0.245

the metabolism was already very low. I have seen a number of cases where the amount of N in the urine before the fast days was between 3 and 4 gm., and where this amount was reduced by 0.5 to 1.0 gm., and a prefasting urine N of 2.5 to 2.9 gm. reduced by more than 0.5 gm. on fast days.

This is a much lower urine nitrogen than that reported even after prolonged fasts on normal persons (Tigerstedt, 5.4 gm.;

[1] The N in the feces was not determined, but is estimated as 1 gm. per day. In the data obtained prior to September, 1922, there is a *minus error in the nitrogen intake* due to the fact that the N in the bouillon given the patient was not included in the calculated diets

Luciani, 3 to 4 gm.; Benedict, 6.9 gm.). But Müller and Nebelthau have obtained urine N as low as 1.5 to 3 gm. in emaciated patients on practical fasting.

Unless the diabetic patient is given a high calorie (fat) diet prior to the fast days, the fasting increases instead of decreases the urine nitrogen. We have occasionally fed our diabetic patients on fat only (butter, up to 200 gm. per day). As a rule such fat diet days also show reduced output of urine nitrogen.

As previously noted I have not, in the cases here reported, regularly made determination of the amount of N in the feces, but Dr. Malmros has determinations of N in the feces in six

TABLE VI.

Diabetic Patients.

Patient No.	No. of days in the determinations.	N intake.	N in feces.	N in urine.
124 b	5	2.3	0.9	2.0
124 b	12	3.2 (+0.72)	1.1	4.5
178	5	1.8 (+0.54)	1.1	3.2
177	5	1.1 (+0.22)	0.93	2.4
183	13	1.9 (+0.97)	0.69	4.3
183	3	3.2 (+1.1)	0.51	2.9
184	14	1.9 (+0.28)	0.74	4.9
98 f	3	3.7	0.73	2.8
184	2	600 cc. bouillon.	0.205	4.7
184	2	600 " "	0.23	3.4
183	2	600 " "	0.23	2.9
183	2	200 " "	0.215	2.5

diabetic patients at different periods and on different diets. The data are given in Table VI.

The figures in parentheses for the N intake (in Table VI) represent the calculated amount of N in the bouillon that the patients received.

When the patient has taken the same food always very poor in N we can see that the amount of N in the feces varied between 0.5 and 10.0 gm. per day. This corresponds to a waste of 30 to 70 per cent of the N intake. We know from Rubner's investigations that in normal persons on ordinary diets there is a waste of only 10 per cent, but on diets rich in coarse vegetables there is a waste

of 10 to 30 per cent. It seems quite improbable that a diet so poor in protein as the one here used should lead to this enormous waste of the food nitrogen in the alimentary canal.

It seems more probable that in such cases most of the N of the feces is of endogenous origin. Rieder found even on protein-free diets 0.54 gm. (3 days) and Roehl 0.43 gm. (4 days). Rubner, on an N intake of 1.36 gm., found 1.39 gm., of N in the feces (2 days). Landergren, on an N intake of 0.27 gm. (almost pure fat), found 1.14 gm. of N in the feces (3 days), and on a diet of carbohydrates (0.75 gm. of N intake) there was 1.0 gm. of N in the feces (4 days). On a more mixed diet (an intake of 1.3 gm. of N in the food) Landergren found 0.45 gm. of N in the feces (13 days). Salomon and Wallace found on diets free from protein 0.64 and 0.38 gm. of N in the feces (3 days).

There is thus a fairly close agreement between the different investigators. On diets very poor in protein or quite free from protein the N in the feces amounts to 0.5 to 1.0 gm. per day.

The literature is meager on the fecal N in complete fasting. Fr. Müller has reported varying figures, 0.45 gm. (4 days), 0.22 gm. (4 days), and 0.17 gm. per day (6 days). The figures from Cetti's fast were 0.32 to 0.35 gm. per day; from Breithaupt's fast, 0.116 gm. per day (Lehmann, Mueller, Munk, Senator, and Zuntz). Benedict in his case of 31 days fast did not secure satisfactory data on the fasting feces. His figures show only the value of 0.035 gm. of N per day in feces.

Thus, we see that in fasting the N in the feces is generally not more than 0.2 to 0.3 gm. per day, and may be as low as 0.1 gm. per day or less. But on diets free from protein the fecal N rises to 0.4 gm. This may, in part, be due to the N in the digestive secretion (endogenous), and to increased bacterial action. But we cannot at present determine what part of the fecal N is of endogenous origin.

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A STUDY OF A MILK-COAGULATING ENZYME OF SOLANUM ELAEAGNIFOLIUM.

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The berry of a certain weed known locally as "horse-nettle" has been used as a rennet substitute for the coagulation of milk in New Mexico and Arizona.[1] The plant was identified by Professor K. M. Wiegand, of the Department of Botany of the New York State College of Agriculture, as *Solanum elaeagnifolium* Cav. (1).

The widespread occurrence of plant chymases (2) suggested the presence of a chymase in the berry of *Solanum elacagnifolium*. We therefore undertook: (1) To ascertain the presence of a chymase in the plant named; (2) to determine its distribution in the plant and its abundance and activity at different seasons and stages of growth; (3) to investigate its properties if found; (4) to test for other proteolytic enzymes, sometimes associated with chymases; (5) to study in greater detail the differences, if any, between animal rennin and plant chymases, with reference to the mechanism of coagulation of casein; and (6) to determine the toxicity, if any, of the raw material and extract.[2]

The first three problems are dealt with in this paper, the others in forthcoming papers.

An early series of experiments, performed on material collected in the late fall, after the weeds had dried in the field, has been re-

[1] The writer is indebted to Mr. L. E. Freudenthal, of Las Cruces, N.M., for this information; and to Mr. Ph. Freudenthal, of Solomonsville, Ariz., and to members of the Staff of the State College at Mesilla Park, N. M., for the raw material used in these experiments.

[2] Cases of poisoning have been reported to the writer, in which consumption of the weed or its berries had been suspected.

ported briefly (3). The method of separation of the enzyme was outlined and some of its properties were described. In the course of further work upon new material available at every stage of the plant's growth, the method of extraction was perfected in detail, most of the early observations were confirmed, some modified, and others added.

Separation of Chymase.

After repeated trials it was found that *complete extraction could be obtained without the use of excessively large amounts of extracting medium when Gerber's procedure (4) was modified as follows:* The material was dried thoroughly at 40°C., ground finely, mixed with enough 5 per cent sodium chloride solution to make a semiliquid mash, and macerated for 24 hours at 5–10° (about 5 drops of the essential oil of mustard had been added to a liter of the saline, as preservative). The outlet of a conical percolating vessel was stoppered with a 1-hole stopper through which a glass tube was passed; rubber tubing and a screw pinch-cock were attached. A small tuft of cotton was pressed gently down the neck of the percolator until it reached the stopper, then it was weighted down with a small amount of quartz sand, and moistened with a few drops of water. The percolator was half filled with mash, a circle of filter paper was placed on top of its contents in order to prevent the floating up of the solid material, and the vessel was filled with saline solution. After the first drops of the extract appeared, the rate of flow was adjusted to about 60 drops a minute. The first portions were poured back into the percolator. Extraction continued until the last portion contained no chymase.

The extract was generally clear. In a few instances it had to be clarified by centrifuging. Salting-out, as in Gerber's procedure, was employed to obtain a few enzyme preparations. The preparations were purified, dialyzed, and dried. However, it was found consistently that the albumin fraction was very small. Its separation, therefore, seemed unnecessary, and it was possible to obtain a solid preparation of the enzyme by an adaptation of Van Slyke and Cullen's method for the preparation of urease (5), which possessed the advantages of greater directness, convenience, and rapidity.

The liquid extract was poured into 30 volumes of redistilled acetone, with constant shaking. *The nature of the precipitate which formed was observed in a preliminary trial* with small volumes of extract and acetone. The precipitate was generally fine, amorphous, and settled rapidly, leaving a clear supernatant liquid.

Sometimes the precipitate was coherent and gummy in consistency, presumably due to some variation in the composition of the sample extracted. In all such cases, *addition of sodium chloride to the main bulk of the liquid extract before pouring into the acetone secured normal precipitation.* The amount of sodium chloride to be added was checked by trial precipitations. Generally not more than 2 per cent was required.

The precipitate was transferred to a Büchner filter, sucked dry, then dried *in vacuo* over sulfuric acid. The powder obtained went easily into solution in water. When kept in small vials completely filled with the preparation, its activity has remained unimpaired for 6 years.

The solutions of the solid preparation were at first dialyzed against frequent changes of distilled water for several days, essential oil of mustard being used as preservative. It was found later, however, that in dilutions of 1:100 and higher the effect of the salt was negligible. This finding is in harmony with Gerber's observation that sodium chloride accelerates the action of several animal and plant rennins when present in concentrations up to about 50 mg. molecules per liter (about 0.29 per cent), then retards coagulation when the concentration of salt is increased further to about 2,000 mg. molecules per liter (about 11.7 per cent).

Method of Testing the Activity of Preparations of Chymase.

Tubes containing 4.5 cc. of milk[3] were brought to the temperature of the bath, and 0.5 cc. of the enzyme solution was added. The tubes were securely stoppered with rubber stoppers, placed in a specially devised holder, and rotated so as to mix the contents by slow inversion (at the rate of not more than 60 inversions a minute, in order to avoid the possibility of inactivation of the enzyme by rapid agitation). Only a small portion of the tube emerged from the water at any time, and the level of the mixture in the tube always remained well under the level of the water

[3] In order to secure a substrate of uniform composition, the author followed Blum and Fuld (6) in using a prepared milk. Merrell-Soule's dry skimmed milk was employed in a dilution of 1:9. 0.2 cc. of a 20 per cent calcium chloride solution was added to 100 cc. of the "prepared milk." Blum and Fuld's and Wohlgemuth's (7) proportions of calcium chloride were found to be excessive.

bath. At the higher temperatures, when steaming of the water bath would interfere with ease of observation, sodium chloride or calcium chloride was added to the bath. The time of coagulation was taken when fine particles of coagulum were first seen clinging to the walls of the emergent portion of the tube. It was possible in this manner to check within 1 per cent or better on long coagulations (requiring 2 minutes or longer). On short coagulations (less than 2 minutes) the divergences were relatively greater, amounting sometimes to 3 per cent. The time was taken with a stop-watch, counting from the moment of addition. In view, however, of the errors of observation, fractions of a second are not stated.

The temperature of the water bath was constant to within $\pm 0.05°$.

EXPERIMENTAL OBSERVATIONS AND DISCUSSION.

Well authenticated observations by many investigators have shown chymases from different sources to differ in behavior under different conditions influencing their action.

Effect of Dilution.—Segelcke and Storch (8) first observed that the coagulation time was inversely proportional to the quantity of rennet used. Subsequent investigations have established the validity of the law of Segelcke and Storch, for animal rennin. Koettlitz's claim (9) that rennin follows Schütz's rule has not been corroborated.

Briot (10) and Bang (11) have pointed out that parachymosin does not follow the rule of Segelcke and Storch. Gerber (12) attributes this discrepancy between the behavior of parachymosin and rennin to the choice of an unsuitable temperature range. Among the plant chymases, however, Gerber himself found (13) that papayotin and the enzyme of *Ficus carica* do not obey the rule of Segelcke and Storch.

The writer's data, obtained with the chymase of *Solanum elaeagnifolium*, are in general agreement with the rule of Segelcke and Storch (with a modification to be stated presently), except at the higher temperatures, where appreciable destruction of enzyme during the test results in greater apparent length of time required for coagulation. The results of a typical test are presented in

Table I. The concentrations used were between 1 part of the solid preparation in 10,000 parts of milk and 1 in 100,000 parts.

Similar results were obtained in a large number of tests.

A general agreement with the law of Segelcke and Storch is indicated at 37°, 47°, and 55°, by the relative constancy of the product $[E]. t. 10^3$. The consistent, although slight, decrease of the product shows, however, that as the dilution is increased the activity is increased relatively (something analogous to the increase of the degree of dissociation of electrolytes). A further study of this detail is contemplated.

TABLE I.

Temperature.	$[E] . 10^3$	t	$[E] . t . 10^3$
°C.		sec.	
37	0.1000	400	40.0
	0.0667	605	40.3
	0.0450	858	38.6
	0.0300	1,256	37.6
	0.0200	1,863	37.2
47	0.0450	290	13.05
	0.0300	425	12.75
	0.0200	643	12.86
55	0.045	204	9.18
	0.030	300	9.00
	0.020	418	8.36
81.5	0.050	63	3.15
	0.010	600	6.00

$[E]$, concentration of enzyme; t, coagulation time.

At 81.5° the law of Segelcke and Storch does not hold. As the dilution is increased the relative activity is diminished, presumably due to rapid destruction of the enzyme.

Temperature Relations.—It will be observed further that as the temperature rises from 37° to 47° the rapidity of coagulation is increased about threefold. $[E]. t. 10^3$ at the concentrations 0.000045, 0.000030, and 0.000030 changes from 38.6 to 13.05, from 37.6 to 12.75, and from 37.2 to 12.86, respectively.

A slower increase of activity is observed between 47° and 55°, and between 55° and 81.5°.

The Optimum Temperature.—Rennet is assumed to act best at about body temperature. According to Fuld, its optimum is at 45° (14).

Plant chymases have very generally a high optimum temperature. Gerber (15) finds *pastel* chymase acting best at about 85°.

The data obtained with one of the author's solid preparations (used in a dilution of 1:20,000) are shown in Table II. Similar results were obtained with other preparations.

It would have been inadvisable to use higher dilutions in order to produce long coagulations, because of greater destructive effect upon the enzyme. On the other hand, the short coagulation times allow only an approximate estimate of an optimum point, which seems to be about 80-85°.

TABLE II.

Temperature.	Time.	Temperature.	Time.
°C.	sec.	°C.	sec.
72	74	79	56
73	73	82	53
74.5	68	83	52
75	67	85	57
78	57	87	78

Resistance to Heat.—The resistance to heat depends on the degree of dilution. The more dilute the solution of the enzyme, the greater its thermolability.

Gerber discusses the resistance of chymases to heat in relation to the normal environment of the plants in which the enzymes are found as well as to specific factors. Those being subjected, while in the living organisms, to greater changes of temperature are assumed to be the more resistant. In general, plant chymases are more resistant than animals chymases. For instance, *pastel* chymase, after being heated to about 83°, retains much of its activity.

A solution of a solid preparation of the chymase of *Solanum elaeagnifolium*, 1:1,000, was distributed into tubes, 0.5 cc. in each. After having been held for 15 minutes at the temperature specified, 4.5 cc. of milk previously heated to 40° were added to each

test-tube, and the series of test-tubes was placed in the water bath at 40°. The activities of the samples thus treated, compared with the activity of a sample kept at the room temperature and used as a standard, are given in Table III.

Traces were found in another tube subjected to 90° for 15 minutes by using a modification of Morgenroth's method (16). Complete destruction of the enzyme had apparently taken place at 100°.

Action at Low Temperature.—Rennin does not coagulate milk at 0°. That some action does occur is well known, and this fact is indeed at the basis of Morgenroth's method for determination of activity of rennin preparations and of gastric juice. Laqueur (17) has suggested that the viscosimetric method offers a means of observing the phenomena of coagulation at low temperatures.

TABLE III.

Held at.	Coagulation time.	Ratio of activity.
°C.		
25	380 sec.	1.000
40	408 "	0.932
50	435 "	0.874
60	555 "	0.685
70	960 "	0.396
80	After 2 hrs.	About 0.050.
90	Not within 8 hrs.	0.000 (?)
100	" " 8 "	0.000

Coagulation by plant chymases at 0° has been reported repeatedly. Gerber (18) relates curdling at 0° to the calcium content of milk, the tendency to coagulation at 0° being the greater the greater the calcium content. However, papayotin produces coagulation at 0° even in calcium-poor milk.

Tubes of milk were placed at 0° for 144 hours, with various concentrations of the chymase up to 1:50. No coagulation was observed.

Dialysis Experiments.

Dialysis having been employed as a part of the procedure for the purification of plant chymases, experiments were undertaken to test the effect of dialysis upon the author's preparations.

In numerous cases the action of an enzyme is dependent upon the presence of coenzymes which are dialyzable. Furthermore, Jacoby (19) observed the diffusion of rennin through the amnionic membrane.

A liquid extract was used in three experiments and a solution of a solid preparation (1:1,000) in one. Parchment paper dialyzing bags were employed. In order to avoid errors in the estimation of the potency of the preparations after dialysis that might be due to possible adsorption of enzyme by the membrane, the dialyzing bags were filled with a portion of the preparation used. The rate of adsorption being most rapid at the beginning of the process, 15 minutes contact was deemed sufficient. The dialyzing bags were then emptied, drained for 5 minutes, and filled with fresh 50 cc. portions. The dialyses were carried out in the cold (5–10°C.), against about a liter of 2 per cent sodium chloride, changed

TABLE IV.

Preparation.	Coagulation time.			
	Not dialyzed.		Dialyzed.	
	min.	*sec.*	*min.*	*sec.*
Liquid extract (1:1)	12	40	13	00
			12	30
			13	25
Solid preparation (1:2,000)	5	20	5	40

at intervals of 8 to 18 hours, and continued for 72 hours. Mustard oil was used inside and outside the dialyzing bags. The contents of the bags were then removed into 100 cc. volumetric flasks, the bags being drained for 5 minutes, as before. The flasks were filled to the mark and comparisons were carried out with portions of liquid extract and with a solution of a solid preparation (1:2,000) used as controls. 0.5 cc. of the preparation was tested with 4.5 cc. of milk. The temperature was maintained at 37°.

The results were as given in Table IV.

The differences between the observed potencies of the chymase preparations before and after dialysis are probably due to mechanical losses or to slight destruction of the enzyme in the course of dialysis. They cannot be attributed to diffusion of a coenzyme, or of the enzyme itself across the dialyzing membrane.

Action of the Chymase upon Raw and Boiled Milk.

The second stage of the process of coagulation, *i.e.* the precipitation of calcium paracaseinate formed in the first stage by the action of rennin on calcium caseinate, is subject mainly to the influence of two factors. It is accelerated in the presence of calcium ions, and it is retarded by lactalbumin and lactoglobulin, which act as protective colloids.

Briot (20) has asserted that the coagulation of fresh milk by the enzyme of *Ficus carica* is retarded by an antirennin, and explains the readier coagulation of heated milk by assuming that the antienzyme has been destroyed.

But Gerber (21) and Gerber and Berg (22) prove that albumins and globulins from various sources have an inhibiting effect; they cite evidence of the progressive destruction of the so called antibody between about 65° and 80°, which is also the range of temperature within which lactalbumin and lactoglobulin coagulate, and they maintain correctly that it is not necessary to assume the existence of an antienzyme in raw milk. Their view is in harmony with the well known protective action of emulsoid colloids upon suspensoids.

It may be assumed, therefore, that when milk is boiled the retarding effect upon the coagulation of casein is eliminated. But in the process calcium salts are also precipitated.

Animal rennin coagulates boiled milk less readily than raw milk. Plant chymases differ widely. Some are stated to coagulate raw milk, and others fail to coagulate it; some are stated to coagulate boiled milk without the addition of calcium chloride, others require the addition of the salt, resembling animal rennin in that respect. Dependent, as the second stage of coagulation of casein is, upon the calcium, albumin, and globulin content of the milk, it seems that the differences in the composition of natural milk have not been sufficiently considered in the statement of the differences between plant and animal chymases.

With a large number of samples of freshly drawn winter milk, the chymase of *Solanum elaeagnifolium* coagulated all the tubes of raw milk, and about 50 per cent of the tubes of boiled milk. In the others, coagulation either failed to take place, or was retarded. It seems, in view of the inconstancy of composition,

and, therefore, of the behavior of raw milk, that a casein solution of standard composition would be more suitable for the study of the differences, if any, between animal and plant chymases, at different H ion and calcium concentrations, and with additions of proteins as protective colloids.

Activity of Preparations of Various Parts of the Plant.

The berries, flowers, flower buds, leaves, stem, and roots of the plant were ground separately and many samples of each were extracted. After grinding the material as described, 1 gm. was extracted with 30 cc. of 5 per cent sodium chloride solution for 96 hours in the presence of the essential oil of mustard. The extracts represented all portions of the plant through the entire growing season. In addition, berries present on the same plant in different stages of ripening were divided in another series of experiments into three lots—green, yellow-green, and yellow—representing successive stages.

All tests yielded negative results for all extracts except those of the berries, and in the case of those the variations were irregular and could not be ascribed to season or to the maturity of each batch.

Gerber has stated that plant chymases are most abundant during the summer and decrease in the winter.

Prochymase.—In order to establish whether or not the inactive material contained the chymase in zymogen form, samples were extracted with a mixture of 27 cc. of 5 per cent sodium chloride and 3 cc. of 0.1 N hydrochloric acid, a procedure used to activate prorennin. The inactive extracts obtained above were also acidified with 0.1 N hydrochloric acid in the same ratio. Both series were again tested for chymase action, with negative results. No prochymase was found by this method. Other methods of activation remain to be tried.

SUMMARY.

1. The presence of a casein-coagulase (chymase) in the berries of *Solanum elaeagnifolium* is demonstrated and the method of its separation is given.

2. The obedience of the chymase to the law of Segelcke and Storch is shown, but there is apparently a slight increase in relative activity with dilution.

3. It is found that the enzyme has a higher optimum temperature (80-85°) and resists heat better than animal rennin. No coagulation is observed at 0°.

4. The activity of the enzyme is not affected appreciably by dialysis.

5. The enzyme coagulated raw milk; its behavior with boiled milk apparently depended on the composition of the milk, coagulation occurring in some samples, and failing to take place or being retarded in others. It is suggested that reported differences between plant and animal chymases may be due in part to the inconstant composition of the milk used. A casein substrate is suggested.

6. Chymase was found in appreciable amounts only in the berries of the plant. The other portions contained neither chymase nor prochymase. There was no consistent variation in potency of extracts prepared from berries collected at different times during the growing season, or separated according to their apparent ripeness.

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VARIATIONS IN THE RATE OF EXCRETION OF THE ACETONE BODIES DURING THE DAY.*

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In articles by Shaffer (1922) and Hubbard and Wright (1923) it has been suggested that the excretion of the acetone bodies which takes place when diets low, but not very low, in carbohydrate are fed—such excretions as have been lately described by McCann and his coworkers (McCann, Hannon, Perlzweig, and Tompkins (1923))—is caused in part by variations in metabolism which take place at different times of the day. Richardson and Mason (1923) have recently shown that if diabetic patients are fed small meals of constant composition at regular intervals throughout 24 hour periods the rate of the excretion of acetone is fairly constant. It has seemed desirable to inquire into variations in this rate in the case of normal subjects upon diets low in carbohydrates who were receiving their food in three meals, for it is upon data obtained in such experiments that much of the discussion of Shaffer's theories of ketogenesis has been based.

The subjects selected for study were four patients who were receiving the Pemberton (1917) diet for the treatment of arthritis as modified by Wright and Hubbard (1921). It seems probable that the metabolism of these patients can be considered as normal for the purposes of such a study in spite of the slightly abnormal glucose tolerance tests obtained on patients with this disease by Pemberton and Foster (1920), for Hubbard and Wright (1922) have shown that normal and arthritic subjects who are receiving similar diets excrete similar amounts of acetone.

*A preliminary report of the work was read before the Western New York Branch of the Society of Experimental Biology and Medicine in October, 1923 (Hubbard and Wright (1923–24)).

The basal metabolism of each patient was determined by the Benedict portable calorimeter (Benedict (1918)) and the diet fed was calculated to contain sufficient food to maintain metabolic equilibrium (20 to 40 per cent more calories than the basal requirement, depending on the activity of the patient) and sufficient protein to maintain nitrogen equilibrium. These diets were similar to those used in the earlier work (Hubbard and Wright (1922), Hubbard (1923)). It is probable that both in these experiments and in those reported previously the calories fed were not always sufficient for the needs of the subjects, although weight may sometimes be maintained by them for long periods (Hubbard and Wright (1922)), but for the study of variations in the rate of acetone excretion such as are presented below such discrepancies are comparatively unimportant. Each of the diets fed contained between 1 and 2 molecules of ketogenic for each molecule of antiketogenic material as calculated by a slightly modified form of Woodyatt's (1921) formula. Each of the subjects was placed upon the diet for at least a few days before the study was carried out. Not only were the diets kept as constant as was possible from day to day, but, as far as was consistent with the comfort of the patients, they were divided into three meals which contained the same amounts of the foodstuffs. These meals were fed at 8, 12.30, and 6 o'clock. Analyses for the amounts of the acetone bodies were carried out on 24 hour specimens of urine by a technique previously described (Hubbard (1921)) and when the determinations showed that the excretion from day to day was approximately constant eight specimens were collected during a 24 hour period at the times shown in Table I.

The results of the analyses of these specimens are given in Table I and shown graphically in Charts 1 and 2. Besides the studies on the results of the experimental diets the table includes a similar series of determinations on an arthritic subject on a normal diet (Case 1). There was little variation in the concentrations of the acetone bodies in the different specimens from this subject, and it seems probable that they are for the most part within the limits of accuracy of the method. All the subjects on the experimental diets excreted more acetone than did this one, although the differences in the amounts obtained from him and

TABLE I.

No.	Ketogenic ratio.	Time.	Determinations in urine.				
			Volume.	Acetone bodies.			
				In 100 cc.		Amount excreted.	
				A*	B*	All* total.	All* per hr.
			cc.	*mg.*	*mg.*	*mg.*	*mg.*
1	?	7–9	70	0.0	0.0	0.0	0.0
		9–11	295	0.5	0.2	2.1	1.1
		11–1	330	0.4	0.2	1.8	0.9
		1–3	190	0.4	0.2	1.1	0.6
		3–5	210	0.5	0.1	1.3	0.7
		5–7	350	0.4	0.1	1.8	0.9
		7–7	660	0.4	0.3	6.3	0.5
		Sum.				14.4	
		In 24 hrs.	2,105	0.4	0.3	14.6	0.6
2	1:1.1	8–10	190	3.6	3.3	13.2	6.6
		10–12	108	2.2	2.2	4.7	2.4
		12–2	190	3.6	4.0	14.6	7.3
		2–4	117	2.55	2.2	5.4	2.7
		4–6	260	1.7	1.5	8.3	4.2
		6–8	110	1.2	3.9	5.65	2.9
		8–10	98	4.05	2.85	6.75	3.4
		10–8	490	2.0	2.45	21.65	2.2
		Sum.				80.25	
		In 24 hrs.	1,563	2.3	2.45	74.3	3.1
3	1:1.1	8–10	61	1.55	2.75	2.6	1.3
		10–12	92	2.7	2.65	4.9	2.5
		12–2	160	6.95	7.2	22.6	11.3
		2–4	520	1.7	0.2	10.0	5.0
		4–6	350	6.3	3.9	35.7	17.9
		6–8	82	2.3	1.0	2.7	1.3
		8–10	72	19.45	16.5	25.9	12.95
		10–8	156	16.5	28.5	70.5	7.0
		Sum.				174.9	
		In 24 hrs.	1,493	5.5	7.35	191.4	8.0

*Under "A" the concentration of acetone from preformed acetone and acetoacetic acid, and under "B" that from β-hydroxybutyric acid is listed. Under "All" the rate of excretion of the sum of all three acetone bodies is given. Results of the determinations of the acetone bodies are in all cases expressed as acetone.

TABLE I—*Concluded.*

No.	Ketogenic ratio.	Time.	Determinations in urine.				
			Volume.	Acetone bodies.			
				In 100 cc.		Amount excreted.	
				A*	B*	All* total.	All* per hr.
			cc.	*mg.*	*mg.*	*mg.*	*mg.*
4	1:1	7–9	215	1.0	0.1	2.3	1.2
		9–11	360	0.4	0.2	2.2	1.1
		11–1	290	0.7	0.2	2.5	1.3
		1–3	120	0.6	0.6	1.5	0.8
		3–5	580	0.0	0.0	0.0	0.0
		5–7	430	0.7	0.5	5.0	5.0
		7–9	320	0.3	0.4	2.35	1.7
		9–7	570	1.2	0.5	9.9	1.0
		Sum.				25.75	
		In 24 hrs.	2,885	0.75	0.2	24.5	1.0
5	1:1.3	7–9	93	7.8	2.6	7.5	3.7
		9–11	108	4.5	3.5	8.7	4.3
		11-1	230	11.2	7.5	40.6	20.3
		1–3	107	32.8	15.3	51.4	25.7
		3–5	45	44.4	24.1	30.8	15.4
		5–7	46	14.1	5.5	9.0	4.5
		7–9	39	19.5	8.4	10.9	5.5
		9–7	500	7.0	2.5	47.7	4.8
		Sum.				206.6	
		In 24 hrs.	1,168	13.7	4.5	212.0	8.8

from the patient whose diet contained 1 molecule of ketogenic for each molecule of antiketogenic material were very small. To show approximately how much reliance can be placed upon the values found, aliquots of each sample were mixed, and analyses carried out upon the representative 24 hour specimens so prepared. The amount of the total acetone bodies found in this specimen in each case has been compared with the sum of the amounts found in the eight separate specimens. The figures show that the results of the fractional studies can be regarded as fairly accurate.

The studies show that there was considerable variation in the concentration and rate of elimination of the acetone bodies in the different specimens from the subjects on experimental diets, and

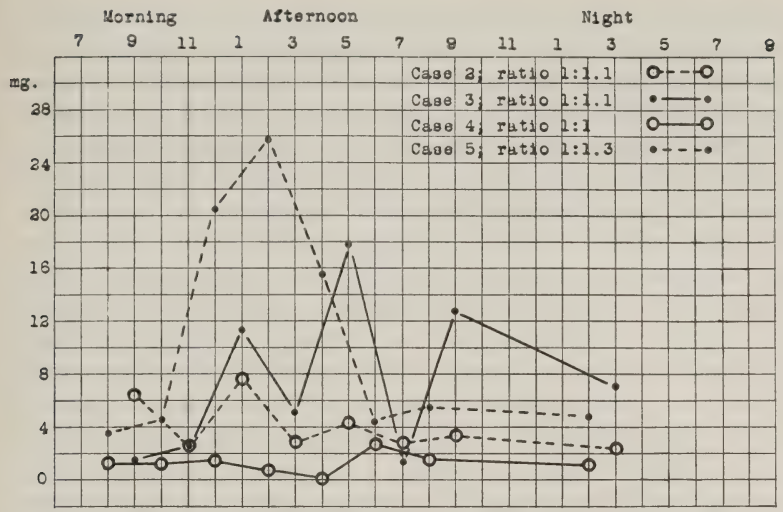

CHART 1. Variations in hourly excretion.

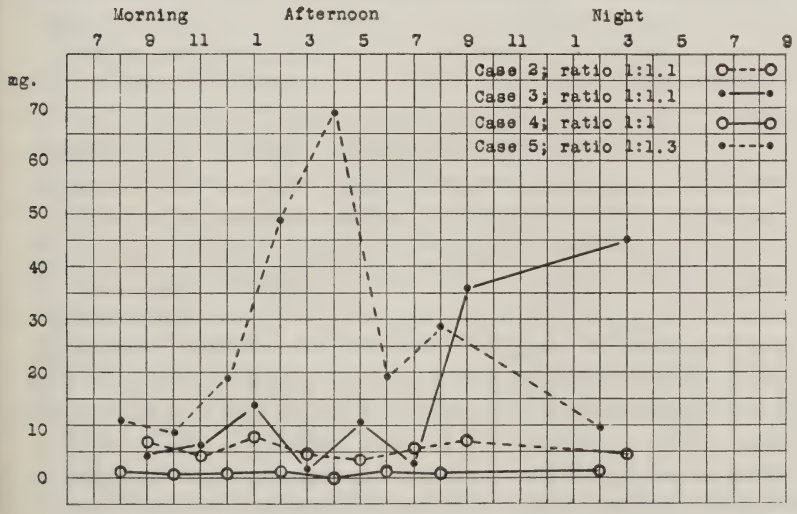

CHART 2. Variations in concentrations.

that practically every specimen from these subjects contained them in at least slightly increased amounts. The results show that these variations did not depend upon variations in urine volume, for in many instances the variations in concentration and rate of excretion were parallel. This can be easily seen from comparing the two charts. In all these cases the excretion at night was lower than in the daytime, and in all but one (Case 3), in which the night specimen of urine measured only 156 cc., there was also a higher concentration in the day than in the night. These results are, it appears to the author, the ones to be expected, for the metabolism of the subjects was higher in the day, but there is a marked difference between them and the findings of Forssner (1909) who, in experiments upon himself in which only protein and fat were eaten in three meals, found the highest rate of excretion at night. Forssner's results can probably be explained as he suggests; the larger part of the protein was usually taken in the first two meals, and the relative amount of fat was higher in the last meal than in the other two.

In general the maximum rates of excretion were found during the periods 2 to 4 hours after the meals, with periods in which the rate was somewhat lower between those in which it was increased. This was not invariably the case. Subject 5, for instance, showed no such alternation of periods of low and high rates of excretion; it seems probable that two high rates of excretion have overlapped to produce the very high rate shown between the hours of 11 a.m. and 5 p.m. A possible explanation of the relationship between the times at which the meals were eaten, and those at which the maximum and minimum excretions of acetone occurred, is that the carbohydrate in the diet was more rapidly assimilated and more promptly burned than was the fat, and that, therefore, the antiketogenic material supplied with each meal was rather promptly used up. Such a suggestion can only be offered tentatively because the relationship between the time of the meal and the time of the maximum excretion of acetone was not always the same.

Whatever may be the cause, the experiments show that rather marked variations in the rate of acetone excretion do take place. As stated above, it has been repeatedly suggested that such variations in acetone excretion, resulting from variations in

metabolism at different times of the day, might account for a part of the acetone found when, according to Shaffer's (1922) theories of ketogenesis, no increased excretion of acetone should be expected. The experiments tend to confirm such an explanation, but they also show that it is not sufficient to account for all the findings, for at least slightly increased amounts of acetone were present in practically all specimens analyzed. It seems probable that variations in the relative amounts of ketogenic and antiketogenic material burned in different parts of the body when a large excess of antiketogenic material is not available would account for the excretion of such small amounts of acetone as were present in some of the specimens. Such a possibility has been suggested by Shaffer (1922) and Hubbard and Wright (1923).

It has been shown in earlier work that the amounts of acetone excreted in a day when diets such as were used in these experiments are fed are not as great as would be expected from a strict application of the theory that 1 molecule of antiketogenic will burn only 1 molecule of ketogenic material. The same fact holds for the results reported. If any subject who was receiving a diet which was expressed by a ketogenic ratio of between 1:1 and 2:1 had continued to excrete acetone throughout 24 hours at the maximum rate found for any period the amount so excreted would not have equalled that required by the theory. Case 3 during the period between 4 and 6 o'clock approached most closely to excreting the amount of acetone so predicted, and in that case the amount found was only one-third of the amount calculated. It seems as though these facts furnished additional evidence against the theory that the "molecule to molecule" ratio most accurately expresses the relationship between antiketogenic and ketogenic material which controls the production of the acetone bodies.

The most important source of error in these studies which could have affected the validity of these calculations—apart from those of a purely experimental character such as the failure of the subjects to eat the diet, and these we believe did not take place—is a discrepancy between the calories ingested and the calories burned in a day. It is difficult to determine just what effect this has upon calculations of the ketogenic balance based upon the diets. Certainly the larger part of the material utilized as a

source of extra energy is the reserve fat of the body. This would serve as source of additional ketogenic material, and would increase the difference between the amounts of acetone found and calculated. Richardson and Mason (1923) have shown that a combustion of carbohydrate, not contained in the diet, but drawn from the glycogen reserves of the body, can be demonstrated in diabetic patients in whom the disease is of long standing. This would serve as a source of antiketogenic compounds for which allowance is not made in the discussion, and would reduce the differences found between the calculated and observed rates of acetone excretion. Boothby and Barborka (1924) have recently shown that in normal subjects an equilibrium is rather quickly established between carbohydrate ingested and metabolized. The preliminary period in these experiments was almost certainly long enough to establish an approximate equilibrium, for, as already stated, all the subjects were excreting acetone at approximately a constant rate before the experiments were performed, and in two instances (Cases 2 and 3) the fore period on a low carbohydrate diet had lasted about a month. It does not seem improper, therefore, to conclude that nearly all the antiketogenic material burned by them in a day was included in the diet, and that the food which was needed above that supplied came from the body fat. If this is accepted the discrepancies between the amounts of acetone predicted and those found were even greater than was stated in the preceding paragraph, if, as is not improbable, the amount of food used was insufficient for the needs of the subjects.

CONCLUSION.

Three subjects who received diets containing between 1 and 2 molecules of ketogenic for each molecule of antiketogenic material divided into three meals excreted small amounts of the acetone bodies which varied greatly in quantity at different times in the day. The variations did not depend upon differences in the rate at which urine was excreted. The maximum rate at which the substances were eliminated seemed to bear a relationship to the time at which the meals were eaten. If these subjects had continued throughout the 24 hours to excrete acetone at the maximum rate observed they would not have excreted such amounts as are required by the theory that 1 molecule of antiketogenic material

can bring about the complete combustion of only 1 molecule of ketogenic material. It seems probable that such variations in the rate of acetone excretion result from variations in metabolic conditions which account for much of the acetone found when diets low, but not very low, in antiketogenic material are fed.

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STROPHANTHIN.

V. THE ISOMERIZATION AND OXIDATION OF ISOSTROPHANTHIDIN.

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When strophanthidin is boiled with sodium hydroxide solution, it dissolves because of saponification. The assumption of Feist,[1] however, that two lactone groups were involved in the reaction was shown to be erroneous by the titration experiments of Windaus and Hermanns[2] which established definitely the presence of only one lactone group in the molecule. When the alkaline solution is reacidified, however, strophanthidin itself cannot be regained, but an isomeric substance, isostrophanthidin (strophanthidinic acid lactone of Feist, isocymarigenin of Windaus and Hermanns), is obtained which like strophanthidin was shown to possess the formula $C_{23}H_{32}O_6 \cdot \frac{1}{2} H_2O$.[3] The formation of this substance was believed by Feist to be due to lactonization on a hydroxyl group different from that involved in the lactone group of strophanthidin, an assumption which was accepted by Windaus and Hermanns and which one of us was inclined to adopt.[4] However, as we have since found, this view is not correct.

It has been the experience of previous workers and of ourselves that only a portion of the material is recoverable as isostrophanthidin after strophanthidin is saponified and that owing to the decomposing effect of boiling alkaline solutions, yellow, amorphous, alteration products form a considerable proportion of the material obtained when the mixture is acidified. Even when very dilute alkali has been used, and such as permits of

[1] Feist, F., *Ber. chem. Ges.*, 1898, xxxi, 534; 1900, xxxiii, 2088.

[2] Windaus, A., and Hermanns, L., *Ber. chem. Ges.*, 1915, xlviii, 993.

[3] Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1922, liv, 261.

[4] Jacobs, W. A., *J. Biol. Chem.*, 1923, lvii, 554.

the correct titration of the substance, we have not been able to recover more than 50 per cent of the starting material as isostrophanthidin. In the course of a more detailed study of this reaction, it was found that strophanthidin could be quantitatively converted into isostrophanthidin by the action of an excess of methyl alcoholic potassium hydroxide solution at ordinary temperature. By pouring the alkaline mixture into ice water, the unsaponified portion was obtained directly in a form which, for reasons to be discussed below, we have designated as α-isostrophanthidin. From the alkaline mother liquor containing that portion, the saponification of which was unavoidable, α-isostrophanthidin was recovered by acidification to Congo red. Strong acidification to Congo red we have always found necessary in order to induce the rapid and complete lactonization of isostrophanthidinic acid as the corresponding acid should be called. The conversion of strophanthidin into isostrophanthidin, therefore, cannot have involved saponification of the lactone group. The lactone groups in both strophanthidin and isostrophanthidin are identical.

The isomerism of these substances is apparently not one of epimerization. In a number of instances there were differences noted in the chemical behavior exhibited by these substances towards reagents which indicated a difference in structure other than that due to stereoisomerism alone. Although strophanthidin is converted into a dihydro compound with palladium and hydrogen, isostrophanthidin was recovered unchanged when exposed to the same conditions. When an attempt was made to isomerize dihydrostrophanthidin with alcoholic alkali, it was recovered unchanged. This suggests that the transformation of strophanthidin into isostrophanthidin may involve a shift in the olefinic linking. Apparently the carbonyl group does not function in the rearrangement since isostrophanthidin also yields an oxime. The identical oxime was obtained by rearrangement of strophanthidin oxime with alcoholic alkali. Although isostrophanthidin is altered by alcoholic hydrochloric acid, attempts to isolate crystalline derivatives analogous to the ethylals of mono- and dianhydrostrophanthidin were unsuccesful. And in their behavior towards oxidizing agents other differences were noted. Efforts to obtain a substance from isostrophanthidin analogous

to the acid, $C_{23}H_{30}O_7$, formed by oxidizing strophanthidin in acetone solution with permanganate led to no tangible result. In fact, isostrophanthidin in acetone solution is much more gradually attacked by permanganate than the former.

On the other hand, the behavior of isostrophanthidin towards aqueous hypobromite and permanganate has led to observations which demonstrate the difficulty and complexity of structural studies with this group of substances. When isostrophanthidin is shaken with aqueous sodium hypobromite, it is gradually dissolved. From the mixture two acids were obtained which analysis showed to be $C_{23}H_{32}O_7$ and $C_{23}H_{30}O_7$, respectively. The latter, characterized by the sparing solubility of its ammonium salt, was obtained in smaller amount and proved to be a monobasic lactone acid which readily gave an ester. The latter did not form an oxime or a benzoate. It is isomeric with the acid obtained from strophanthidin with permanganate in acetone solution, the ester of which, however, yielded a benzoate. Contrary to the latter acid, the saponified acid from isostrophanthidin proved to be quite stable towards permanganate.

Of greater immediate interest is the acid, $C_{23}H_{32}O_7$, which preponderated. This acid which we shall call α-isostrophanthidic acid is also a monobasic lactone acid which easily forms an ester. The ester yields both an oxime and a benzoate. It is formed apparently by the action of 1 mol of hypobromite which furnishes 1 mol of oxygen. At the moment, with the facts available, the interpretation of the course of this reaction is difficult.

α-Isostrophanthidic acid in dilute ammoniacal solution rapidly consumes an amount of permanganate equivalent to an additional mol of oxygen with the formation of a new acid, $C_{23}H_{32}O_8$, in which still another carboxyl group is produced. This acid is a dibasic lactone acid and readily forms a dimethyl ester which no longer forms an oxime, but yields a benzoate. It was then found that this acid may be directly obtained by the action of permanganate on α-isostrophanthidin after the latter has been dissolved in dilute sodium hydroxide at room temperature. The amount of permanganate required was the equivalent of 2 mols of oxygen. The acid produced by hypobromite, therefore, represents an intermediate stage in the formation of the acid,

$C_{23}H_{32}O_8$, from isostrophanthidin. The latter acid we shall designate as α-isostrophanthic acid.

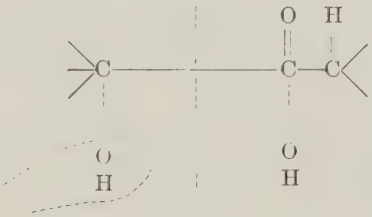
At first sight, this observation seemed at variance with previous observations, namely that the oxidation with permanganate of both strophanthidin and isostrophanthidin after saponification gave rise to the so called strophanthic acid of Feist for which Windaus and Hermanns and one of us had accepted the formula, $C_{23}H_{30}O_8$, and which distinctly differed in physical properties from α-isostrophanthic acid. More recent analytical results with the former acid and its derivatives have shown that this formula must be changed to $C_{23}H_{32}O_8$ and that it is isomeric with α-isostrophanthic acid. We believe that the name strophanthic acid for this acid should be changed to β-isostrophanthic acid since it is in all probability epimeric with the α compound and is a derivative of isostrophanthidin.

The condition which precedes the formation of the β acid instead of the α compound is a stereoisomerization produced in isostrophanthidin by the boiling alkali formerly used for the preliminary saponification of both strophanthidin and isostrophanthidin. It was then found that if α-isostrophanthidic acid is subjected to the action of boiling sodium hydroxide solution, a similar isomerization occurs since the resulting mixture when oxidized with permanganate yielded in the main β-isostrophanthic acid. If the solution containing the salt of β-isostrophanthidic acid is acidified to Congo red, an acid was recovered which proved to be a mixture of both α- and β- isostrophanthidic acids since on oxidation a mixture of α- and β-isostrophanthic acids was produced. Attempts to prepare the β acid in pure form have thus far been unsuccessful, owing to the greater solubility of the β form and also, as we are inclined to believe, to the fact that on the acid side, reversion of the β into the α form apparently occurs. This point, however, will require further investigation.

Although in the case of isostrophanthidin, β-isostrophanthidin is also produced under the influence of alkali, the isolation of the pure substance has proved difficult for similar reasons. We are convinced that the isostrophanthidins studied in the past, and all of which were obtained by saponification of strophanthidin in boiling alkaline solution, were mixtures of varying amounts of the α and β forms. In our hands, different

preparations prepared by this method have varied considerably in crystalline form and melting points, whereas the substance resulting from the action of alcoholic alkali on strophanthidin has always given at once an anhydrous high melting (255–257°) form which is α-isostrophanthidin. This has been borne out by the homogeneity of the acid produced from it by permanganate which, at present, is the only criterion which we possess.

Contrary to either the isostrophanthidins or the isostrophanthidic acids, neither α- nor β-isostrophanthic acid, except for saponification, is altered by boiling sodium hydroxide solution. In this pair of acids the carbonyl group contained in the other pairs of substances is no longer present. The isomerization observed in the latter cases may occur on an asymmetric carbon atom containing a hydrogen atom which adjoins the carbonyl group. Although the second mol of oxygen is unquestionably used for the conversion of the carbonyl group into carboxyl, the adjoining asymmetric carbon atom then becomes α to a carboxyl. This may be represented as follows:



EXPERIMENTAL.

α-Isostrophanthidin.—10 gm. of strophanthidin were dissolved in a cold solution of 2.4 gm. of potassium hydroxide in 100 cc. of methyl alcohol and the solution was allowed to remain at room temperature for 1½ hours. After this time, isomerization was complete and the alkaline mixture was poured into 4 volumes of ice water. A voluminous crystalline precipitate of isostrophanthidin separated, but owing to partial saponification of the lactone group about 60 per cent of the material remained in solution. In order to recover this, the solution was made distinctly acid to Congo red. On gentle warming to about 40–50° the second fraction was obtained which proved to be identical in

all respects with the first portion. Recrystallized from 50 per cent alcohol it formed lustrous scales which melted with effervescence and slight preliminary sintering at 255–257°. Contrary to the substances prepared by saponification of strophanthidin in boiling dilute alkali this material is anhydrous. It dissolves easily in acetone, less readily in alcohol and chloroform, and is but sparingly soluble in ether. Contrary to strophanthidin the substance is tasteless at first, but slowly develops a slightly bitter taste towards the back of the tongue. It gives, in atypical manner, a slowly developing Liebermann cholestol reaction although in concentrated sulfuric acid, it develops the same brown-red solution produced by strophanthidin.

$[\alpha]_D^{22} = 34$ ($c = 0.0993$ in methyl alcohol).

$C_{23}H_{32}O_6$. Calculated. C 68.27, H 7.98.
Found (*a*). " 68.29, " 8.05.
(*b*). " 68.54, " 8.29.

All attempts to reduce isostrophanthidin gave negative results. For this purpose a methyl alcoholic solution with colloidal palladium and an acetic acid solution with an active palladium black were used, conditions under which strophanthidin fairly readily yielded the dihydro compound. In acetone solution isostrophanthidin is more slowly affected by permanganate than strophanthidin and from the reaction product no crystalline substance was isolated. When isostrophanthidin was shaken at ordinary temperature with an excess of normal sodium hydroxide solution, it dissolved completely within an hour whereas with strophanthidin under the same conditions, a much longer time was required. The resulting solution when acidified with acetic acid remained clear and all attempts to isolate the crystalline acid were without result although it can be salted-out as a gum which very gradually lactonizes. Hydrochloric acid precipitated the acid at once as a gum which gradually changed to the lactone, a process greatly facilitated by warming the mixture.

When strophanthidin was saponified by boiling in equal parts of 0.1 N alkali and alcohol for 1 hour and the neutralized solution concentrated to small volume, acidification of the mixture to Congo red yielded an amorphous precipitate which was warmed to complete lactonization. When recrystallized from 50 per cent

alcohol radiating groups of delicate needles mixed with platelets were obtained. This melted with effervescence at 239–241° although melting points as low as 221–223° were observed. This material, contrary to the substance obtained by the former method, is a hydrate containing $\frac{1}{2}$ mol of water of crystallization. We are convinced that the different appearance and melting point are not only due to solvent of crystallization but to contamination with varying amounts of β-isostrophanthidin. Evidence of this was obtained by the oxidation of such material with permanganate since the resulting isostrophanthic acid was not homogeneous.

α-Isostrophanthidin Oxime.—2 gm. of isostrophanthidin, 1 gm. of hydroxylamine hydrochloride, and 3 gm. of sodium acetate were refluxed for 2 hours in 20 cc. of alcohol. When a portion of the solvent was removed and the mixture was carefully diluted with water, needles of the oxime slowly crystallized. Recrystallized from 25 per cent alcohol, flat needles were obtained which sintered at 233° and melted with effervescence at 236°.

$[\alpha]_D^{26} = 75$ ($c = 1.000$ in pyridine for the air-dry substance).

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4.

$C_{23}H_{33}O_6N \cdot 2H_2O$.	Calculated.	H_2O 7.92.
	Found.	" 7.10.

Anhydrous Substance.

$C_{23}H_{33}O_6N$.	Calculated.	C 65.83, H 7.93.
	Found (*a*).	" 65.91, " 7.97.
	(*b*).	" 66.15, " 7.99.

A substance, identical in all its properties with the above oxime, was obtained directly from strophanthidin oxime by the action of methyl alcoholic potassium hydroxide as in the preparation of isostrophanthidin. This conclusion was controlled by analysis, rotation, and mixed melting point.

Oxidation of Isostrophanthidin with Hypobromite.

In the oxidation of isostrophanthidin with hypobromite the course of the reaction has been found to be influenced considerably by the procedure used. In our first experiments isostrophanthidin as such was used directly, but when the method had been found for the almost quantitative conversion of strophan-

thidin into α-isostrophanthidin, no attempt was made to isolate the isostrophanthidin and the following method was employed.

α-Isostrophanthidic Acid.—50 gm. of strophanthidin were digested at ordinary temperature in a solution of 12 gm. of potassium hydroxide in 500 cc. of methyl alcohol. The solution was diluted with water and the methyl alcohol removed by careful distillation under diminished pressure during which the volume was not allowed to become less than 500 cc. After diluting with water to 1,250 cc. the solution of the salt of α-isostrophanthidinic acid was treated with 40 gm. of bromine dissolved in 750 cc. of 4 per cent sodium hydroxide. The clear yellow solution was left at room temperature for 45 minutes during which the color gradually faded. On addition of 100 cc. of acetic acid and particularly when the solution was heated a copious deposition of lustrous crystals occurred. After cooling, the deposit was collected with water. The yield of this fraction was 22 gm. Additional fractions of apparently identical material were obtained by acidifying the mother liquor to Congo red and by allowing the solution to stand. The deposition of substance slowly continued for several days as if, on the one hand, lactonization was gradually completed or as if a gradual reversion had occurred of some β acid which might have been present owing to a certain degree of isomerization due to the action of alkali on isostrophanthidin. The total yield was 35 gm. The mother liquor, even after seeding and long standing, gave no indication of the presence of the acid, $C_{23}H_{30}O_7$, to be described below.

Recrystallized from 95 per cent alcohol, the acid forms lustrous leaflets which are difficult to filter. It melts with effervescence at 231–233° and contains 1 mol of water. This melting point was observed with fair regularity, although on several occasions specimens of apparently unquestionable purity and of the same composition were found to melt with effervescence from 10–30° below this point. The methyl ester to be described below exhibited a similar variable melting point. The acid is easily soluble in acetic acid, less readily soluble in alcohol and acetone and practically insoluble in chloroform or ether. In sulfuric acid it gives at first a brown-orange solution which deepens to a deep brown with an olive reflex.

$$[\alpha]_D^{23} = -16.3 \ (c = 0.674 \text{ in 95 per cent alcohol}).$$

Titration of the acid showed the presence of the lactone group. 0.2043 gm. of anhydrous substance required 5.05 cc. of 0.1 N alkali against phenolphthalein. One equivalent for $C_{23}H_{32}O_7$ is 4.85 cc. 0.2060 gm. of anhydrous substance was refluxed for 1 hour in 20 cc. of 0.1 N alkali and then titrated back. 10 cc. of alkali were consumed. The amount calculated for 2 equivalents is 9.80 cc. Although more stable towards boiling alkali than isostrophanthidin, when stronger alkali is used the solution turns yellow due to the formation of alteration products which are precipitated as yellow flocks on acidification. The isomerization which also occurs will be discussed later.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4.

$C_{23}H_{32}O_7 \cdot H_2O$. Calculated. H_2O 4.27.
Found. " 4.18.

Anhydrous Substance.

$C_{23}H_{32}O_7$. Calculated. C 65.68, H 7.68.
Found (*a*). " 65.59, " 7.53.
(*b*). " 65.41, " 7.69.

α-Isostrophanthidic Methyl Ester.—This was obtained by the action of diazomethane on a suspension of the acid in dry acetone. The ester which incompletely separated was collected with ether. Although this material as first obtained melted slowly from 238–243°, when recrystallized from methyl alcohol it formed needles which sintered at 218° and melted with effervescence at 223°. However, when this was again recrystallized from acetone it melted at 210–212°. On another occasion, recrystallization from methyl alcohol gave crystals which sintered at 242° and melted at 249°. Although in these cases the retention of small amounts of solvent which could variously effect the melting point is not excluded from tests which were made, this seemed unlikely. Isomerization also appears improbable since even apparently identical conditions of recrystallization gave variable results. Observations of a similar character have already been made in the case of other members of this class of compounds[5] and reference of such variability to polymorphism has been made.

The methyl ester is readily soluble in acetone and chloroform, less readily so in alcohol, and practically insoluble in benzene and ether. In sulfuric acid it gives an orange-brown solution with yellow fluorescence.

[5] Windaus, A., and Weil, K., *Z. physiol. Chem.*, 1922, cxxi, 68.

$[\alpha]_D^{20} = -15$ ($c = 1.000$ in chloroform).

$C_{24}H_{34}O_7$.	Calculated.	C 66.33, H 7.89.
	Found (*a*).	" 66.47, " 7.90.
	(*b*).	" 66.37, " 7.90.

α-Isostrophanthidic Methyl Ester Benzoate.—This was prepared in the usual manner by benzoylation of the ester in pyridine solution. For recrystallization the sparingly soluble benzoate required a large volume of methyl alcohol. It formed glistening, short, stout prisms which melted and effervesced at 230° after preliminary sintering.

$C_{31}H_{38}O_8$.	Calculated.	C 69.11, H 7.12.
	Found (*a*).	" 68.80, " 7.06.
	(*b*).	" 69.15, " 7.19.

α-Isostrophanthidic Methyl Ester Oxime.—This was prepared from the ester by heating several hours in methyl alcohol with hydroxylamine hydrochloride and sodium acetate. Recrystallized from methyl alcohol it formed short, pointed platelets or rods which first sintered and then melted with effervescence at 263°.

$C_{24}H_{35}O_7N$.	Calculated.	C 64.10, H 7.85.
	Found.	" 64.04, " 7.91.

The Acid, $C_{23}H_{30}O_7$.—25 gm. of powdered isostrophanthidin were shaken at room temperature in a solution of 25 gm. of bromine, in 1 liter of 2 per cent sodium hydroxide solution. Solution was complete within 45 minutes. 50 cc. of acetic acid were added to the mixture. An initial turbidity or slight amorphous precipitate was soon followed, when the mixture was stirred and rubbed, by a copious deposit of isostrophanthidic acid. The filtrate was acidified strongly to Congo red with hydrochloric acid. On standing, crystallization slowly proceeded, aided by stirring. This fraction consisted of a mixture of additional quantities of isostrophanthidic acid and a new acid, $C_{23}H_{30}O_7$. The separation of this material continued for a week or more. After collection of the combined fractions with water, the separation of the two acids was accomplished by taking advantage of the sparing solubility of the ammonium salt of the acid, $C_{23}H_{30}O_7$. For this purpose, the mixture was treated with a small volume of concentrated

ammonia. A large amount of the sparingly soluble salt remained undissolved. The separation of the latter was completed by the addition of an equal volume of 40 per cent ammonium sulfate solution. The collected salt was washed with 20 per cent ammonium sulfate. On acidification of the filtrate, the dissolved isostrophanthidic acid was recovered. The combined fractions of this acid, when recrystallized from alcohol, were 7 gm., or considerably below that given by the first procedure described. This was in part due to the further oxidizing effect of the excess of bromine used. When in later experiments the bromine was reduced to two-fifths of the amount used above, or to 50 per cent more than required for 1 mol of oxygen, the yield was considerably improved. The yield of the acid, $C_{23}H_{30}O_7$, however, seems to be determined by other factors as yet undetermined.

The ammonium salt of the new acid was dissolved in hot water and precipitated with acetic acid. 6.3 gm. were obtained. When the acid is allowed to separate slowly by the acidification at ordinary temperature of a dilute solution of its salt, it forms aggregates of needles which melt at 230° and contain 0.5 mol of water of crystallization. When, however, the precipitation occurs from the hot solution or by dilution with water of the hot alcoholic solution of the acid itself, it forms small rectangular leaflets which melt at 254–257° and are anhydrous. In sulfuric acid the acid forms at first a faint yellow solution which very gradually deepens on standing to orange, then brown, and finally after a day or more to an olive. The presence of the lactone group was shown by titration of the anhydrous substance.

0.2008 gm. required 4.90 cc. of 0.1 N sodium hydroxide solution when titrated against phenolphthalein. Calculated for $C_{23}H_{30}O_7$ for 1 COOH group is 4.80 cc. 0.2008 gm. was refluxed for 1 hour with 20 cc. of 0.1 N alkali and titrated back. Calculated for 2 COOH, 9.6 cc. Found 10.0 cc.

Aside from saponification, no evidence of alteration of the acid in boiling alkaline solution was observed. On acidification with acetic acid little tendency to lactonize was shown and from more concentrated solutions the dibasic acid described below was obtained. Acidification to Congo red and gentle warming rapidly regenerates the lactone acid. In alkaline solution it is quite stable to permanganate at ordinary temperature.

$[\alpha]_D^{23} = -69$ ($c = 1.007$ in 95 per cent alcohol).

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4.

$C_{23}H_{30}O_7 \cdot \frac{1}{2} H_2O$.	Calculated.	H_2O 2.10.
	Found.	" 2.01.

Anhydrous Substance.

$C_{23}H_{30}O_7$.	Calculated.	C 65.99, H 7.23.
	Found (*a*).	" 65.87, " 7.16.
	(*b*).	" 65.92, " 7.16.

The Dibasic Acid.—1.1 gm. of the acid were saponified in 25 cc. of 2 per cent sodium hydroxide solution by heating for $\frac{1}{2}$ hour. When acidified with acetic acid pointed, broad, flat needles of the dibasic acid separated which effervesced at 183° after preliminary sintering. It does not give a sparingly soluble ammonium salt.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4.

$C_{23}H_{32}O_8 \cdot 1\frac{1}{2}H_2O$.	Calculated.	H_2O 5.83.
	Found.	" 5.72.

Anhydrous Substance.

$C_{23}H_{32}O_8$.	Calculated.	C 63.26, H 7.40.
	Found.	" 63.04, " 7.44.

The Methyl Ester.—The lactone acid in dry acetone suspension readily yielded an ester with an ethereal solution of diazomethane. The fraction first obtained by boiling off the ether formed long, stout plates which melted at 253–256°. Here again the melting point showed great irregularity. When recrystallized from acetone the same melting point was observed, but occasionally with the same material samples were obtained from acetone which melted 10–20° lower and very unsharply. When methyl alcohol was used as solvent a similar difficulty was experienced. As a means of detecting any possible isomerization as an explanation for this variability the substance was examined for mutarotation. In chloroform solution at 4° no change in rotation was observed.

$[\alpha]_D^4 = -59.5$ ($c = 9.351$ in chloroform).

On attempting to benzoylate the ester in pyridine solution it was recovered unchanged. Likewise, other acylation experiments and attempts to form an oxime were unsuccessful.

$C_{24}H_{32}O_7$.	Calculated.	C 66.63, H 7.46.
	Found (*a*).	" 66.72, " 7.52.
	(*b*).	" 66.45, " 7.52.

Oxidation of α-Isostrophanthidic Acid with Permanganate.

α-Isostrophanthic Acid.—1 gm. of α-isostrophanthidic acid was dissolved in 50 cc. of water by the addition of a drop or two of ammonia. On the addition of 5 per cent permanganate the reagent was fairly promptly used up and after 5.5 cc. had been added or sufficient for 1 mol of oxygen the solution remained purple. Acidification of the filtrate with acetic acid caused the rapid separation of glistening 4-sided leaflets with rounded corners. Recrystallized by addition of water to the hot alcoholic solution it formed long, thin, broad pointed platelets which, when air-dried, melted at 232–234° with effervescence although the melting point was found to vary with individual preparations and the rate of heating. The acid is readily soluble in alcohol and but sparingly so in water. In sulfuric acid it gives at first a yellow color which deepens through orange to a stable, deep, wine-red color. This reaction is identical with that given by β-isostrophanthic acid (strophanthic acid). Titration proved this acid to be a dibasic lactone acid. 0.1097 gm. of air-dried substance required 4.9 cc. of 0.1 N alkali. When boiled for 1 hour with an excess of 0.1 N alkali, 2.4 cc. of alkali in addition were consumed or 7.3 cc. in all. Calculated for $C_{23}H_{32}O_8 \cdot H_2O$, 2 COOH 4.7 cc.; 3 COOH 7.05 cc. This acid is much more stable towards alkali than either isostrophanthidin or isostrophanthidic acid since after boiling for 1½ hours in 4 per cent sodium hydroxide solution the acid was recovered unchanged.

$[\alpha]_D^{20} = -8$ (c = 1.000 in methyl or ethyl alcohols).

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4.

$C_{23}H_{32}O_8 \cdot H_2O$.	Calculated.	H_2O 3.97.
	Found.	" 4.04.

Anhydrous Substance.

$C_{23}H_{32}O_8$.	Calculated.	C 63.26, H 7.40.
	Found (*a*).	" 63.00, " 7.39.
	(*b*).	" 63.42, " 7.47.

On thermic decomposition the acid loses approximately 1 mol of CO_2 and the resulting amorphous substance still possesses acid properties. This fact suggests that the acid is possibly a malonic acid although such speculations are for the present unwarranted.

0.1808 gm. air-dry substance when heated at 200° gave 0.0156 gm. CO_2.

$C_{23}H_{32}O_8 \cdot H_2O$. Calculated. CO_2 9.68.
Found. " 8.62.

α-Isostrophanthic acid was also obtained directly from isostrophanthidin as follows: 3 gm. of isostrophanthidin were shaken at room temperature in 50 cc. of 2 per cent sodium hydroxide solution. Solution was complete within an hour. 35 cc. of 5 per cent permanganate or the equivalent of about 2 mols of oxygen proved sufficient for complete oxidation. The filtrate on acidification with acetic acid slowly deposited characteristic leaflets of α-isostrophanthic acid in excellent yield. The substance melted with effervescence at 232–234°.

$[\alpha]_D^{30} = -7$ ($c = 1.000$ in 95 per cent alcohol).
Air-Dry Substance. Found. H_2O 3.90.
Anhydrous Substance. " C 63.30, H 7.47.

When an isostrophanthidin was used for the oxidation which had been obtained by saponification of strophanthidin in boiling dilute alkali as in the method used for titration of strophanthidin, the oxidation product was an obvious mixture.

α-Isostrophanthic Dimethyl Ester.—The ester was prepared from an acetone suspension of the acid with diazomethane. Recrystallized from methyl alcohol it forms rosettes of long platelets or separate 6-sided platelets which melt at 224–225° with slight preliminary sintering and are soluble in alcohol, acetone, and chloroform, and very sparingly so in ether. The ester did not give an oxime.

$[\alpha]_D^{26} = -12$ ($c = 1.008$ in chloroform).
$C_{25}H_{36}O_8$. Calculated. C 64.66, H 7.82, $(OCH_3)_2$ 13.40.
Found (*a*). " 64.53, " 7.75, " 13.69.
(*b*). " 64.64, " 7.80.

α-Isostrophanthic Dimethyl Ester Benzoate.—The ester was benzoylated in pyridine solution. When the mixture was poured into acid an oil separated which was extracted with ether. The residue from this crystallized when treated with ligroin. Recrystallized from methyl alcohol it formed needles with square ends which melted at 201–203°. It is soluble in benzene and acetone and less readily so in alcohol and ether.

$C_{32}H_{40}O_9$. Calculated. C 67.57, H 7.10.
Found. " 67.36, " 6.99.

Oxidation of α-Isotrophanthidic Acid and α-Isostrophanthidin after Isomerization with Alkali.

A.

2 gm. of α-isostrophanthidic acid were refluxed for 1 hour in 200 cc. of 0.1 N NaOH. After cooling, the slightly yellow solution was treated with an amount of permanganate solution in slight excess of that corresponding to 1 mol of oxygen. The neutralized and concentrated filtrate when acidified to Congo red and allowed to stand 24 hours slowly deposited a crust of crystals which was accelerated by rubbing. The collected crystals proved to be a mixture of α- and β-isostrophanthic acid, the latter preponderating. These were separated by fractional crystallization of the crude substance from dilute alcohol. The first and last fractions consisted of characteristic delicate needles which melted with effervescence at 277–279° and otherwise corresponded in all properties with the β-isostrophanthic acid (strophanthic acid) described in a former communication.

$[\alpha]_D^{25} = -26$ ($c = 1.000$ in methyl alcohol).
$C_{23}H_{32}O_8$. Calculated. C 63.26, H 7.40.
Found. " 63.50, " 7.34.

Like the α compound this acid loses 1 mol of CO_2 on thermic decomposition and the residue dissolved in dilute ammonia.

0.2009 gm. substance lost at 260° 0.0196 gm. CO_2.
$C_{23}H_{32}O_8$. Calculated. CO_2 10.08.
Found. " 9.75.

The intermediate fraction melted and effervesced at 233–236° and proved identical with α-isostrophanthic acid.

$[\alpha]_D^{22} = -8$ ($c = 1.010$ in methyl alcohol).
Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4.
$C_{23}H_{32}O_8 \cdot H_2O$. Calculated. H_2O 3.97.
Found. " 3.90.
Anhydrous Substance.
$C_{23}H_{32}O_8$. Calculated. C 63.26, H 7.40.
Found. " 63.05, " 7.43.

Since the above experiment indicated that isomerization of α-isostrophanthidic acid had occurred on boiling with alkali and since this obviously was not quite complete under the conditions used, an attempt was made to isolate the resulting acid after more vigorous treatment with alkali.

10 gm. of the α acid were boiled under a reflux condenser in 250 cc. of 2 per cent sodium hydroxide solution for 1½ hours. During this process, the solution rapidly turned yellow and deepened considerably in color owing to partial decomposition. The cooled solution when acidified with hydrochloric acid immediately deposited a voluminous yellow amorphous precipitate which was rapidly collected by filtration through bone-black. The clear colorless filtrate was made definitely acid to Congo red. On gentle warming to about 45° and rubbing, colorless crystals rapidly deposited as stout, broad needles which were collected at once. 7.5 gm. of acid were recovered which softened and slowly frothed up at 170–180°. Although as shown below, the acid proved to be a mixture of isomers, no simple method for their separation was found. This was rendered difficult since no criteria aside from oxidation experiments were available to determine absolute homogeneity of material. The specific rotation of the crude material was practically the same as that of the α isomer and the melting points were not characteristic.

$$[\alpha]_D^{30} = -19 \ (c = 0.631 \text{ in } 95 \text{ per cent alcohol}).$$

When recrystallized from alcohol almost half of the material was recovered as lustrous leaflets which melted at 224–226°. Apparently this was still a mixture since oxidation yielded what appeared to be a mixture of α- and β-isostrophanthic acids. In another attempt pure α-isostrophanthidic acid was recovered after recrystallization. The reappearance of this form suggests a possible reversion of the β into the α form under the influence of acids. The presence of β-isostrophanthidic acid was demonstrated by oxidation.

The above recovered crude acid was oxidized with permanganate exactly as in the case of the preparation of α-isostrophanthic acid from α-isostrophanthidic acid after dissolving in water by the aid of a slight excess of ammonia. The equivalent of 1 mol of oxygen was used. The crude acid obtained gave at

once on recrystallization from dilute alcohol characteristic needles of β-isostrophanthic acid (strophanthic acid) which melted and effervesced at 280°.

$[\alpha]_D^{30} = -25$ ($c = 1.010$ in methyl alcohol).
Found. C 63.19, H 7.41.

This was substantiated by conversion into the dimethyl ester which melted at 246–248° and when mixed with β-isostrophanthic dimethyl ester showed no depression.

From the mother liquors of β-isostrophanthic acid fractions were obtained which yielded characteristic crystals of the α isomer.

B.

The preparation of β-isostrophanthic acid from strophanthidin after saponification as described in a former communication may be regarded as indirect evidence of the intermediate formation of β-isostrophanthidin. Nevertheless, the same experiment was repeated with α-isostrophanthidin under the conditions there described. Characteristic needles of β-isostrophanthic acid were obtained which melted with effervescence at 274–277°.

$[\alpha]_D^{30} = -22$ ($c = 1.000$ in methyl alcohol).

There seems to be no question that the strophanthic acid of Feist which melted at 260.8° was a mixture of the α and β isomers.

FAT-SOLUBLE VITAMINS.

XVII. THE INDUCTION OF GROWTH-PROMOTING AND CALCIFYING PROPERTIES IN A RATION BY EXPOSURE TO ULTRA-VIOLET LIGHT.*

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Last year Steenbock and Nelson (1) laid the foundations for an experimental method in which, by the use of ultra-violet light, growth can be used as a measure of the comparative amounts of vitamin A occurring in any food. Their method also allows demonstration, in case of inhibited growth and in the absence of a vitamin A deficiency, as to whether this inhibition is due to lack of the antirachitic vitamin or some other factor. Preliminary data on the use of this method have already been presented (2).

In attempting to use this method for determinations of vitamin A in butter a large series of experiments was run in which eight groups of rats, six animals to the group, were used (3). In each group two animals were exposed to ultra-violet light from a quartz mercury vapor arc, with the expectations that if there were a deficiency of the antirachitic vitamin in the ration, the two irradiated animals would, by their increased growth, reveal such to be the case. It merely remained, then, to find out at what level of butter fat irradiation no longer had any effect. This would be the level at which the butter furnished a sufficiency of the antirachitic vitamin.

Contrary to what was expected, all the animals in each group,

*Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

To protect the interest of the public in the possible commercial use of these and other findings soon to be published, applications for Letters Patent, both as to processes and products, have been filed with the United States Patent Office and will be handled through the University of Wisconsin.

irradiated or not irradiated, irrespective of whether the butter was incorporated in the ration at an 8, 4, 2, 1, 0.5, 0.2, 0.1, or 0.0 per cent level, grew, on the average, equally well. This puzzled us because in dozens of other experiments, and in controls, irradiation had always been found to increase the amount of growth unless the animals gave signs of vitamin A deficiency as indicated by ophthalmia or infections of the respiratory tract. Then, of course, lack of vitamin A was the factor inhibiting growth.

Shortly after these results were obtained, there reached our hands an article by Hume and Smith (4) which appeared to have a bearing on this point. They irradiated glass jars with ultraviolet light and found that rats kept in such jars grew to a larger size than controls. Ozone, well known to be produced by the mercury lamp, was shown not to produce this effect nor did irradiated jars from which the air had been blown out after irradiation. They concluded that "it is the air and not some property impressed on the glass jars which is active." They speak of ionization as being a probable factor.

A little later Goldblatt and Soames (5), studying what they erroneously speak of as fat-soluble A, found that, when the livers of rats in which growth had been induced by irradiation were fed to suitably prepared and rationed animals, growth previously inhibited was restored; with the livers of non-irradiated rats taken under the same circumstances and fed to similar animals this did not take place. They found the results difficult of interpretation. They considered it unjustifiable to postulate synthesis of "fat-soluble A." They suggest that it may be a mobilization from other organs, but conclude that "the correct explanation must await the results of further studies on the nature of the fat soluble growth promoting factor as well as on the site and mode of its action."

It appeared to us that our results might be explained and some idea obtained as to the difficulty experienced by the aforementioned investigators if it were determined, in the first place, whether or not an irradiated animal when put in the cage with a non-irradiated animal is able to influence its growth in any way whatsoever. This suggested itself because in our butter fat experiments irradiated and non-irradiated animals had been kept together in the same cage for the first time—and it was in this instance and in no

other that approximate equality of growth was obtained. In the second place, it appeared justifiable to carry out a test to determine if growth-promoting properties could be conferred upon an ordinary ration by direct irradiation just as Goldblatt and Soames had found that liver irradiated in the living rat was activated. Obviously, positive or negative data on these two points would clarify the situation greatly. Experiments dealing with the first will be published later; experiments dealing with the second form the subject of the present communication.

EXPERIMENTAL.

As data upon one point after another were accumulated the experimental procedure was modified from time to time, but essentially the same technique was followed as in previously published work. White rats and black hooded white rats were used as the experimental animals. They were kept in our standard sized cages provided with false screen bottoms (6) in our rat room in which all the window curtains were drawn except those of northern exposure. This latter precaution was taken even though our experiments were started in early November and completed early in March, when solar radiation, especially such as finds its way through window glass, is virtually impotent. Especial emphasis was placed upon the preparation of our animals, in fact, it was the condition of our animals that first gave us the clue to this method of experimentation and particular care was taken to maintain their condition as uniform as possible. The critical point in the preparation of our experimental animals to allow us to demonstrate what we desired, lay in having them suitably depleted in the antirachitic vitamin and yet stocked with the liberal reserves of vitamin A necessary to prevent early failure from ophthalmia and infections of the respiratory tract.

Our basal stock ration (7) supplemented with milk from stall fed cows kept continually on the same ration, allowed us on the whole to meet the requirements. At times, however, when we were obliged to use poor alfalfa hay in this ration we were forced to supplement the ration temporarily with a small amount of butter fat to keep up the rate of reproduction in our stock necessary to supply us with a sufficiency of experimental animals.

When our young rats weighing 40 to 50 gm. at 21 to 25 days of

age were put upon a ration, free from vitamin A and the antirachitic vitamin, growth usually ceased within 2 weeks as the stores of the antirachitic vitamin became exhausted. With the introduction of the antirachitic factor growth was always restored; without it the animals maintained themselves practically at constant body weight; in either case, with the exhaustion of the reserves of vitamin A, death ultimately resulted from ophthalmia or infections of the respiratory tract.

It is obvious that the decisiveness of our demonstration of the occurrence or non-occurrence of the antirachitic factor lay in the length of time that failure due to lack of vitamin A could be delayed. To prolong this period we initially used a ration of hog millet which contains vitamin A and but little of the antirachitic vitamin; later, we used a synthetic ration supplemented with a small amount of alfalfa, 0.3 per cent cured with special precautions. This we had previously found to carry considerable amounts of vitamin A and but little of the antirachitic vitamin (8). Larger amounts we did not see fit to use because of the danger of the introduction of the antirachitic factor of which rats, on our rations, need but little for normal growth and bone calcification. Our synthetic ration consisted of alcohol-extracted and heated casein 18, salt 40, 4, agar 2, yeast 8, and dextrin 68. The preparation of these has been previously described (1).

For irradiation we used a Cooper-Hewitt quartz mercury vapor lamp with an arc $\frac{3}{4}$ inch long at a distance of 23 inches, run for the most part at a voltage of 120.

Our first experiments were carried out with a ration of hog millet 84, purified casein 12, and salts 40, 4. The ration was made up in quantity and fed to the rats *ad libitum* in tin cups. The irradiated ration was exposed for 10 minutes to the radiations of the quartz mercury vapor lamp in 50 gm. quantities, spread out in a thin layer in a 10 inch porcelain dish. After irradiation it was fed immediately. Unconsumed residues were discarded daily.

The results of this trial are shown graphically in Chart I. In each case a very pronounced stimulation of growth was observed. The experiment was terminated at the end of 7 weeks because by this time all the rats were afflicted either with ophthalmia or infections of the respiratory tract due to insufficiency of vitamin A.

Chart II shows the results obtained in a similar experiment

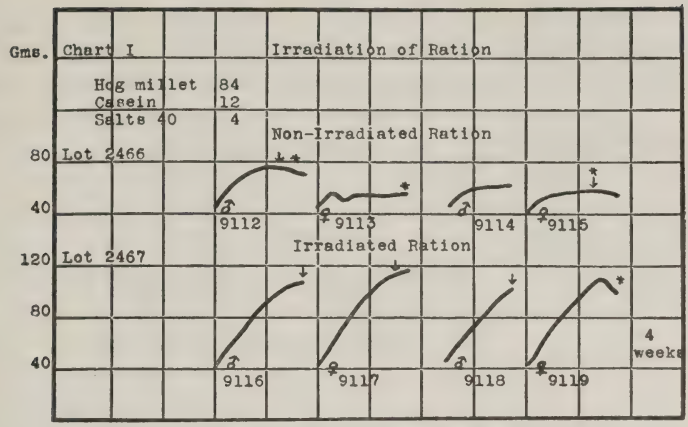

CHART I. Irradiation of a supplemented hog millet ration with a quartz mercury vapor lamp has a very pronounced effect upon growth. The rats were taken at 21 to 23 days of age. The position of the arrow indicates the incidence of ophthalmia and the asterisk the incidence of infections of the respiratory tract.

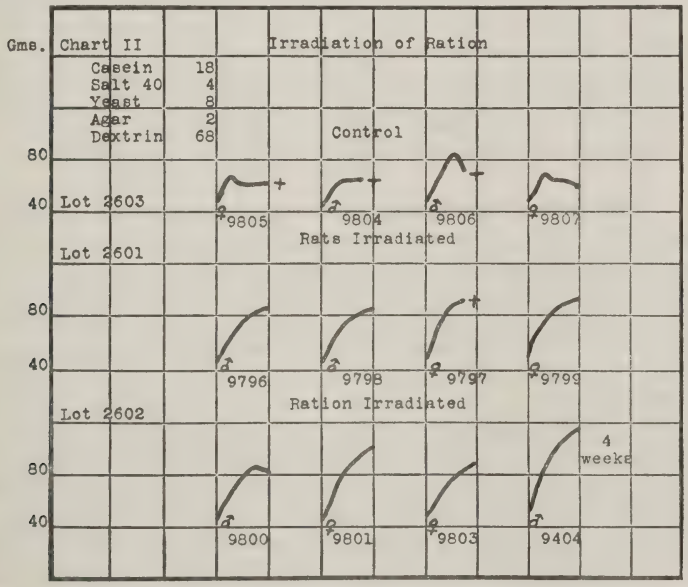

CHART II. Irradiation of a casein, salt, yeast, agar, dextrin ration was also effective in providing a growth stimulus, terminated only by exhaustion of reserves of vitamin A. As this ration carried no intact living plant cells the synthetic action of cells in the production of the vitamin is excluded. These rats were started at an age of 21 to 24 days.

where no vitamin A was furnished in the ration. The early failure of our controls indicates that this group of animals was but very poorly provided with reserves of vitamin A. Yet even here in

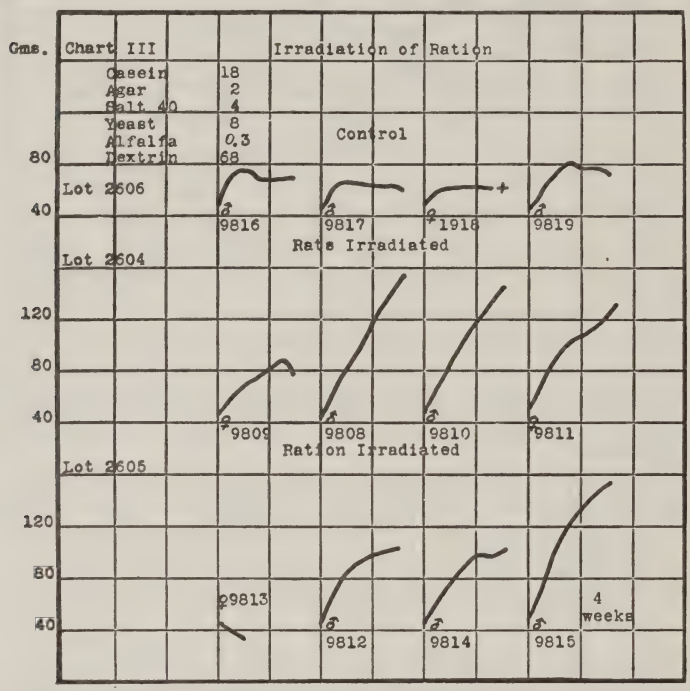

Chart III. When more vitamin A was provided in the ration, irradiation could be continued over a longer time and more decisive results were obtained. The rats were started at 21 to 24 days of age. Unfortunately, Litter 1 was an inferior litter as shown by their growth. Rat 9813 failed rapidly due to a bad pneumonic condition revealed upon post-mortem. Analysis of the femurs for ash gave values of 48.8 and 45.6 per cent with Rats 9817 and 9819; of 55.2, 55.3, 58.6, and 57.5 per cent with Rats 9808, 9809, 9810, and 9811; and of 52.9, 53.2, and 55.8 per cent for Rats 9812, 9814, and 9815, respectively. Increased calcification, therefore, went hand in hand with increased growth. The pronounced growth of Rat 9815 with a difference of 77 gm. in weight from its litter mate control, Rat 9819, together with a difference in ash content of bone of 10 per cent, shows that the effect cannot be looked upon as a matter of temporary stimulation.

4 weeks time it is demonstrated that irradiation of the ration had a decided effect; in fact, it was more effective than exposing the animals to the rays directly. The animals, however, had received

more radiations; they had been irradiated for 10 minutes 6 days out of the week, while the ration had been irradiated 20 minutes daily. The experimental precautions used were the same as in the first series.

As a result of the poor demonstration of the effect of irradiation in the second trial, a third was carried out in which the ration was fortified with respect to its vitamin A content by the addition of 0.3 per cent of alfalfa (Chart III). This had the desired effect; the incidence of ophthalmia or infections of the respiratory tract were delayed for at least 2 weeks except in the case of Litter 1. This was an exceptionally poor litter as the young grew poorly or not at all and autopsy revealed large caseous areas in the lungs, suggesting tuberculosis.

As growth in itself can hardly be taken as sufficient evidence of the existence of the antirachitic factor, we were led to make analyses of shaft bones, usually the femurs or humeri, for total ash content. These were dissected free from muscle and connective tissue and then cleaned by rubbing between folds of cheese-cloth. They were then dried for 2 days in a steam oven at 96°C., extracted thoroughly with alcohol in a Soxhlet for 48 hours, then dried and ashed in an electric muffle furnace. In this series the femurs from two rats of the control group gave an average value of 47.2 per cent ash, four with direct radiation 56.6 per cent, and three on the irradiated ration 53.9.

Further evidence of the effectiveness of irradiation of ration is shown in Chart IV where we also used the alfalfa supplement with our synthetic ration to furnish additional vitamin A. It almost appears that with this the animals were furnished with too much of the antirachitic factor because the control groups grew more than usual. How much supplement to furnish is always a matter of uncertainty because in spite of the precautions taken with our stock it cannot be foretold absolutely how much our animals have in reserve. In no case, however, were we forced to discard any animals or any results for this reason. All our data on growth that had been collected when these experiments were terminated are presented without selection in this paper.

In this series (Chart IV) we were particularly interested with the rôle of air in the activation of the ration because in connection with the therapeutic effect of light attention has been called

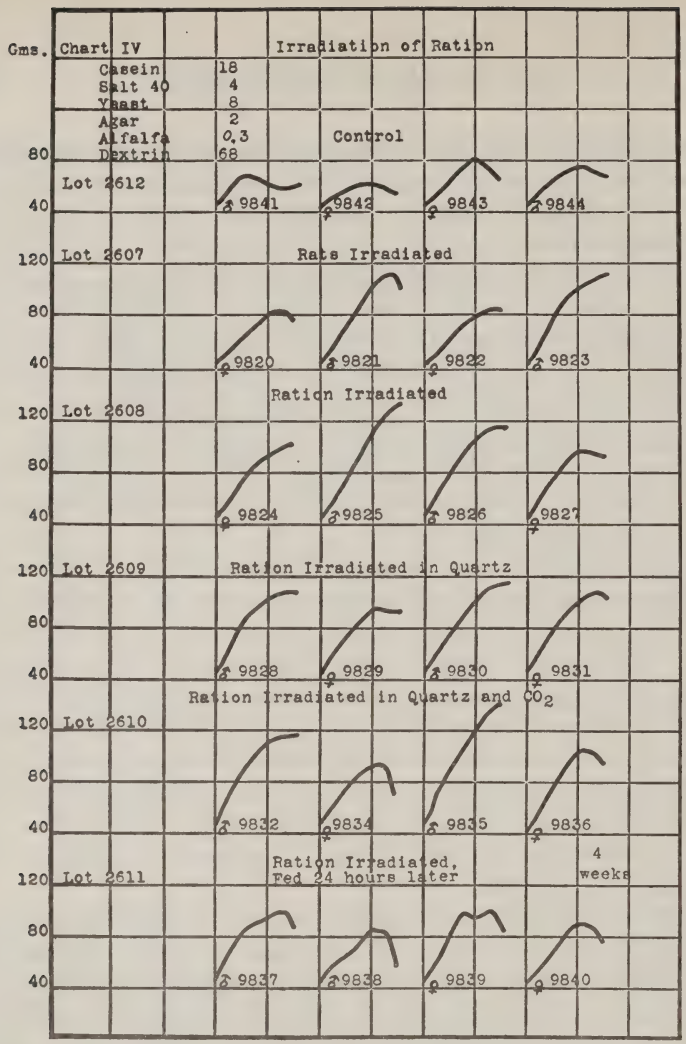

CHART IV. Though the controls show an unusual amount of growth the results show, nevertheless, that irradiation of the ration as carried out in an open porcelain dish, or in a stoppered quartz flask in the presence of air or in a stoppered quartz flask in the presence of CO_2 is effective in activating the ration. Nor is this activation completely dissipated after 24 hours; in fact, it may be questioned if it is reduced at all because when failure ensued signs of vitamin A deficiency were clearly evident. Numbering the litters from left to right—all rats in the same vertical line being from one litter, Litters 1 and 2 were started at 21 days, Litter 3 at 20, and Litter 4 at 23 days.

Analysis of femurs for ash gave values as follows:

Lot 2612; Rats 9841 to 9844; 49.3, 48.2, 47.0, and 48.1 per cent.
" 2607; " 9820, 9821, and 9823; 53.0, 52.1 " 49.4 " "
" 2610; " 9832 to 9836; 54.1, 52.7, 53.8 " 52.8 " "

repeatedly to the rôle of ozone and oxides of nitrogen produced by the irradiation of the elements of air. If these play a rôle in the activation of the ration, less activation should result when the ration is irradiated in the presence of CO_2 and possibly also when the air is limited in quantity. To determine this the ration was irradiated in quartz flasks of 300 cc. capacity, stoppered with a rubber stopper.

In one case the flask containing 50 gm. of ration was exhausted in a vacuum desiccator with a water pump to 16 mm. pressure. CO_2 was then passed in to destroy the vacuum and the process repeated to wash out the air as thoroughly as possible. The flask itself could not be evacuated directly because it was flat bottomed and unable to withstand the pressure. This also prevented us from irradiating the ration *in vacuo*. With the air displaced by CO_2, the stop-cock with which the rubber stopper was provided was turned immediately, and the flask irradiated for 20 minutes. Unconsumed ration residues were discarded daily.

As seen in Chart IV the effectiveness of the irradiation was not reduced by this procedure. We admit that this does not absolutely demonstrate the point that we were after, because, in the absence of analytical control, it is possible that there was still sufficient air in the flask to play an important rôle. Nevertheless, it shows that, within the experimental conditions imposed, air is not an important factor.

The activation of the ration does not appear to be a temporary matter as shown in the last lot of the series. There the rats were fed the ration 24 hours after it had been exposed to the ultra-violet rays for 20 minutes. In the interim the ration had been kept in a glass bottle, stoppered with a rubber stopper. It is true that the rats did not grow so well as in some of the other groups, but neither did the rats exposed to the radiations directly, and failure was postponed until vitamin A deficiency became notable.

Further data on the activation and persistence of the activation of the ration are presented in Chart V. Here we again fed a ration free from the fat-soluble vitamins and, as seen in the chart, we obtained a better control. The first few groups furnish information on the relative transmissability of the activating rays by Pyrex glass as compared with brown bottle glass. 50 gm. portions of the ration, made up in quantity, were irradiated in the one

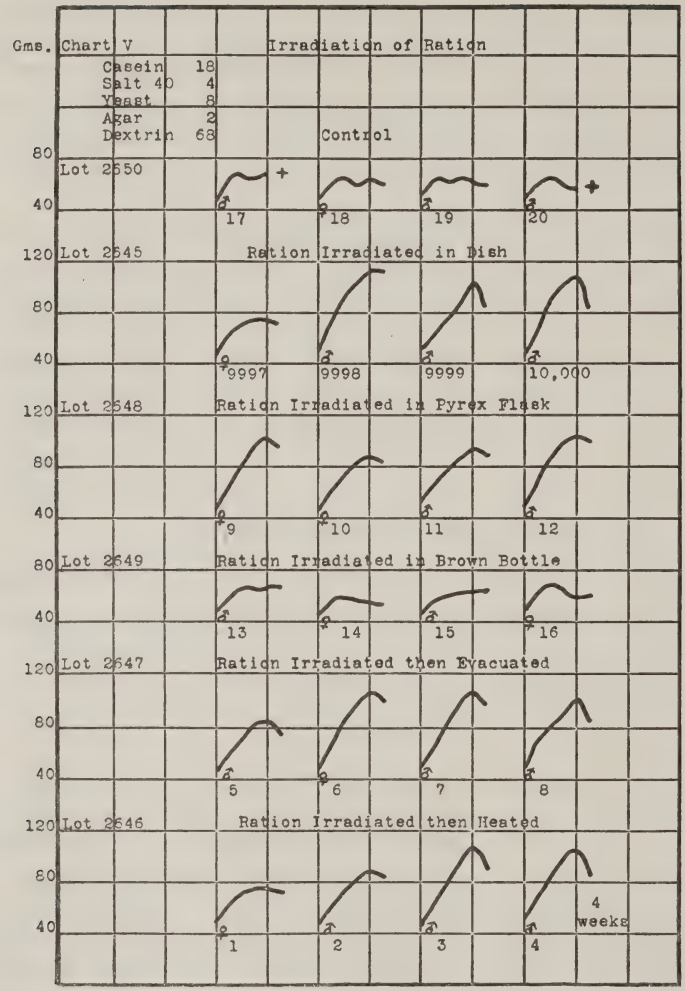

Chart V. Irradiation of the ration in a 500 cc. Pyrex flask in contrast with a brown glass-stoppered bottle did not interfere appreciably with the activation. In harmony with this observation is the fact that Pyrex glass is somewhat permeable to ultra-violet light while brown bottle glass shuts out even the violet. Exposing the activated ration to a vacuum or heating it for 45 minutes at 96° did not destroy the activity. These rats were all taken from four litters as in the previous group. Litters 1, 2, and 3 were taken at an age of 24 days, Litter 4 at 25 days. At the time of terminating the experiment the rats not only varied in weight but in length, showing that actual growth had taken place. From tip of nose to tip of tail Rats 18 and 19 measured $9\frac{6}{8}$ and $9\frac{7}{8}$ inches, respectively; Rats 10 and 11 measured 11 and $11\frac{3}{8}$ inches, respectively.

case in a 500 cc. rubber-stoppered Pyrex flask and in the other case in a 150 cc. rubber-stoppered brown bottle for 20 minutes, the containers being rotated from time to time to expose fresh surfaces. Pyrex, well known to transmit some of the ultra-violet rays, did not prevent the activation in contrast with the brown bottle glass.

The last two groups show that exposing the activated ration to a vacuum or heating it for 45 minutes at 96° did not destroy the activation. In the former case 50 gm. of ration irradiated in a porcelain dish as before were transferred to a beaker and placed in a vacuum desiccator. This was evacuated with a water pump to 16 mm. pressure, then the vaccum was destroyed and the evacuation repeated. The ration was fed to the animals immediately. The heating was carried out in the porcelain dish in which the ration had been irradiated. This was placed in a copper steam-heated oven.

Irradiation of Rat Livers.

With the fact demonstrated that growth-promoting properties can be conferred upon a ration by exposing it to the radiations from a quartz mercury vapor lamp it occurred to us that possibly the results of Goldblatt and Soames (5) could be explained on the same basis. It will be recalled that they irradiated rats, fed the livers of these rats and found them growth-promoting, while livers from non-irradiated rats were entirely inactive. In their experiments there prevailed the possibility that the growth-promoting property was mobilized from other tissues or that it was actually synthesized by cellular activities. The fact that these possibilities were excluded in our previous experiments on irradiation of ration suggested a repetition and elaboration of the experiments of the aforementioned investigators.

Chart VI presents a duplication of the experiments of Goldblatt and Soames with the same results; *i.e.*, the livers of irradiated rats were found to have been activated. The rats used as a source of liver were young rats not over 60 gm. in weight, raised on the same ration as the test animals. The irradiated rats had been exposed to ultra-violet radiations 10 minutes a day, 6 days out of the week. Rats from either group were used as a source of liver as soon as they showed signs of ophthalmia. They apparently were very low in vitamin A content because in the test animals the incidence of ophthalmia was not deferred beyond the time of

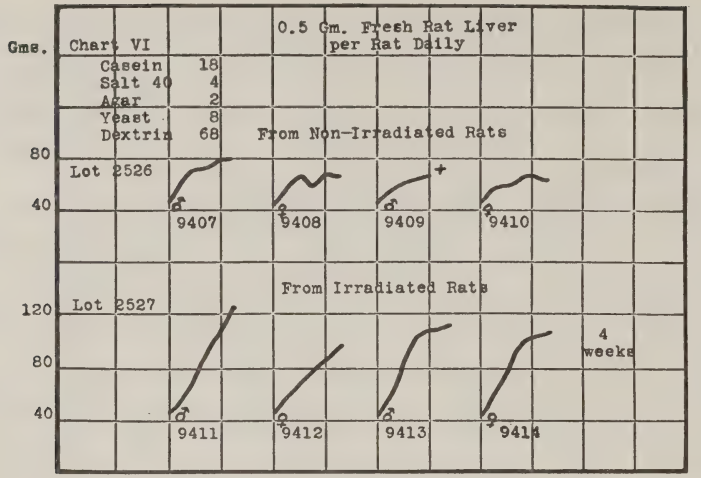

Chart VI. Liver taken from non-irradiated rats is less effective in promoting growth than that from irradiated rats. The percentage of ash in the bones differed markedly. Rats 9407, 9408, and 9410 gave values of 48.6, 50.0, and 49.3; while Rats 9411 to 9414 gave values of 57.8, 57.8, 54.0, and 53.9, respectively.

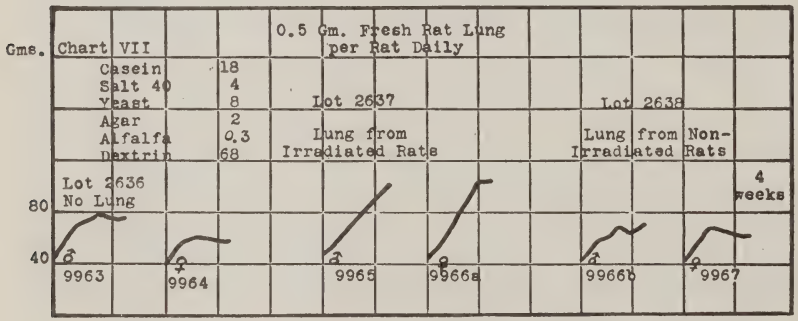

Chart VII. Lung tissue, like liver, varies in its content of the activating agent with the exposure to the ultra-violet radiations. Especial attention was focussed on this experiment in the early belief that if the radiations were growth-stimulating by virtue of their effect upon the air, lung tissue ought to be especially active. Rats 9963 and 9964 had 46.7 and 54.4 per cent of ash in their dried alcohol-extracted femurs; Rats 9965 and 9966 a, 53.9 and 55.0 per cent; and Rats 9966 b and 9967, 46.5 and 49.0 per cent, respectively. All the rats were from one litter taken at 21 days of age.

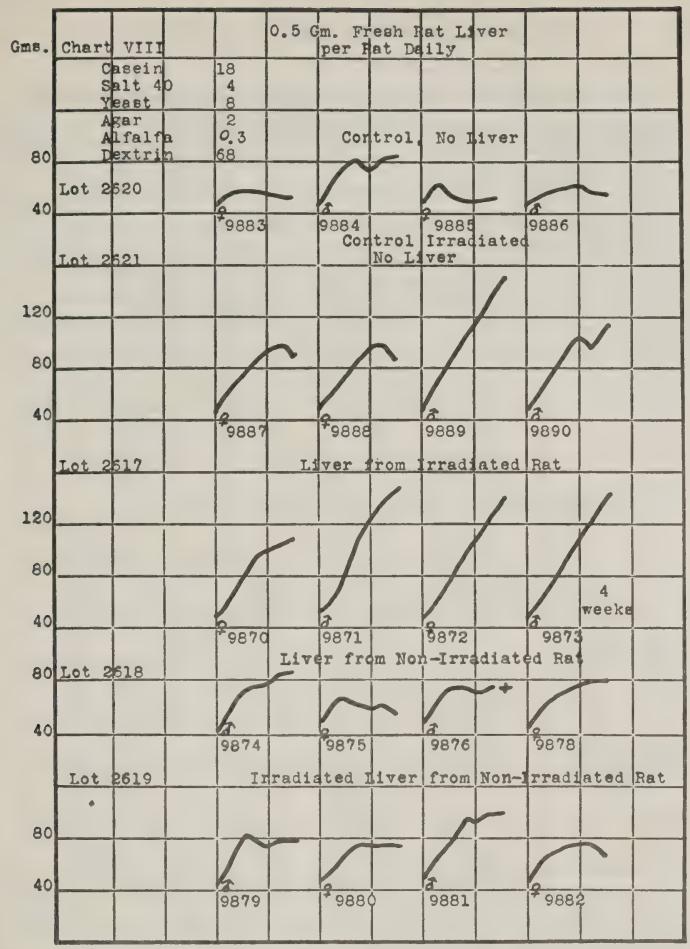

Chart VIII. This chart shows again, as shown in Chart VI, that liver tissue taken from an irradiated rat, in contrast with that from a non-irradiated rat, is active. When liver tissue was removed from a non-irradiated rat and then irradiated, no more growth was obtained with it than with untreated tissue. Bone calcification, however, was increased. Taking rats in order , lot for lot, from left to right, the percentage of ash in the femurs was as follows:

Lot 2620; 44.6, 49.3, 47.1, 43.3
" 2621; 56.1, 52.7, 54.4, 51.8
" 2617; 56.3, 54.1, 56.9, 54.7
" 2618; 47.9, 46.8, 46.9, 44.5
" 2619; 55.1, 51.7, 52.0, 53.1

Apparently, failure of growth is not necessarily accompanied by deficient calcification. The rats were taken from four litters, counting from left to right, Litters 1, 2, and 3 were started at 21 days and Litter 4 at 20 days.

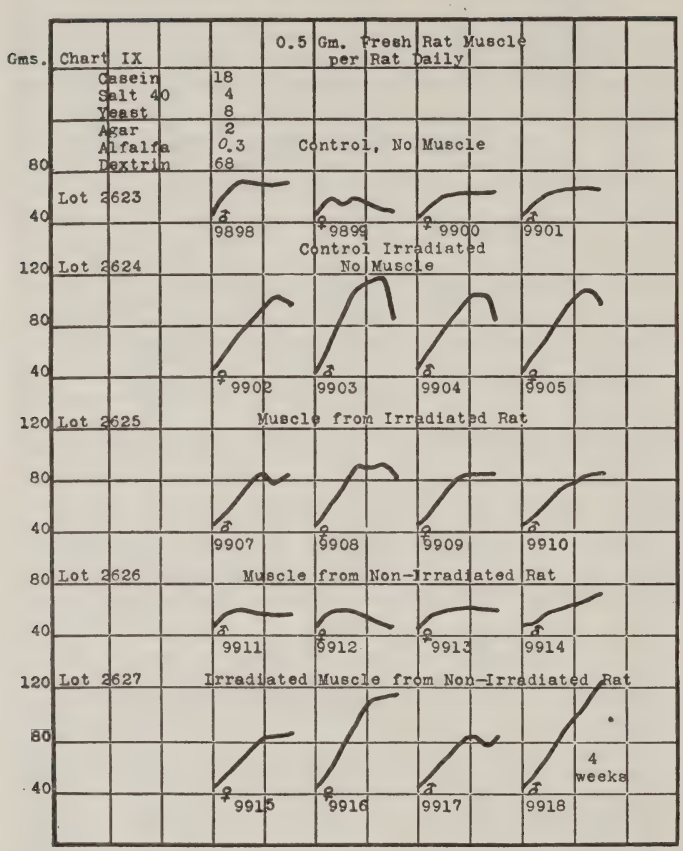

Chart IX. Muscle like lung and liver tissue from an irradiated rat is activated, and when not activated by irradiation of the living animal, it can be activated subsequently. This is shown by increased growth and also increased calcification of bone, when such muscle was fed. Again taking the rats in order from left to right, lot for lot, the percentage of ash in the femurs was as follows:

Lot 2623; 46.6, 47.2, 46.0, 43.7
" 2624; 57.7, 55.0, 53.1, 55.6
" 2625; 52.1, 52.6, 51.6, 51.1
" 2626; 48.5, 45.2, 51.0, 47.8
" 2627; 55.3, 57.6, 49.0, 55.2

The rats were taken from four litters, those in the same vertical line being from one litter. Numbering the litters from left to right Nos. 1 and 3 were started at 23 days of age, No. 2 at 22 days, and No. 4 at 21 days.

its incidence in a control group not receiving any liver. Chart VII shows similar results obtained with lung tissue.

The experiments shown in Chart VIII represent an attempt to determine if non-active liver could be activated by irradiation subsequent to its removal from the rat. To demonstrate this, it was necessary to run sufficient controls so that errors from the side of the test animals as well as from the livers would be excluded. The liver samples, after weighing, were macerated on a glass plate

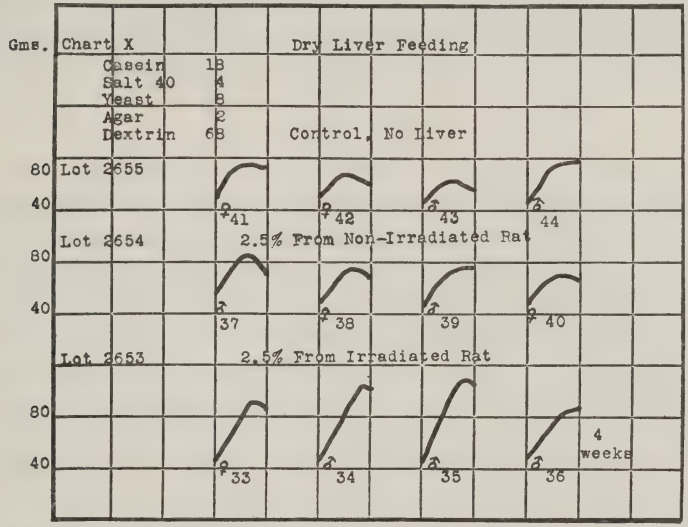

CHART X. Livers from irradiated rats dried at 96°C. for 24 hours and kept in the laboratory for months were still active. The rats used were taken from two litters; the six on the left from one litter; and those on the right from the other. They were started at an age of 23 days.

and then exposed to ultra-violet rays for 20 minutes. They were fed to the test animals immediately afterwards. The chart shows that they were inactive as far as growth is concerned, but analysis of the bones for ash, the results of which are detailed in the legend of Chart VIII, show that calcification was promoted just the same. While the rats from Lot 2618, which had been fed livers from non-irradiated rats, had on the average 46.5 per cent of ash in their bones, those from Lot 2619, getting the same livers but irradiated, had 52.9 per cent. Why these animals should not have grown we cannot explain. Calcification evidently took place independently of growth.

Chart IX shows that muscle tissue subjected to the same treatment is activated beyond question. Not only was growth promoted but calcification as well. Rats in Lot 2626 on non-irradiated liver averaged 48.1 per cent ash and those in Lot 2627 on irradiated liver, 54.3 per cent.

Chart X shows the persistence of the activation in the irradiated rat livers left over trom the earlier experiments. They were dried in a steam oven at 96°C. for 24 hours and then put in glass bottles till a sufficient quantity had been accumulated. Some of the material had stood as long as 60 days on a shelf in the laboratory before being fed. As the chart shows it was still very active.

DISCUSSION.

It is not the intention of the authors to discuss in detail the significance of the data presented in this paper at the present time, because obviously more experimental facts must be obtained. Some time ago we made the assumption that the failure of growth in our young rats in the absence of vitamin A deficiencies was due to the absence of the antirachitic vitamin. We made this assumption because of the parallelism in the resumption of growth upon the administration of cod liver oil, free from vitamin A, with the action of ultra-violet light which is a well known antirachitic agent. This assumption was supported by the fact that with the reinitiation of growth calcification of bone was increased.

The question now presents itself, are we justified in making the further assumption, in view of the fact that growth-promoting and calcium-depositing properties can be imparted to a ration by irradiation with ultra-violet light, that the acceptance of the existence of an antirachitic vitamin upon its former premises is no longer justifiable, granted, of course, that we accept a vitamin as a compound of biological origin.

If such were justified it would simplify our conceptions materially because it has been difficult though not impossible to conceive how two such apparently different agencies, light and vitamin, should have the same effect. It suggests itself that, in ultimate analysis, both light and the antirachitic vitamin may represent the same antirachitic agent—possibly a form of radiant energy. In this connection it is of interest to mention that the authors have conferred growth-promoting properties upon olive oil and lard by irradiation with ultra-violet light. Taken from

three litters and started at 48 to 63 gm. in weight, young rats on the untreated olive oil at the time of writing weigh from 67 to 73 gm.; on the irradiated olive oil 92 to 98 gm. On untreated lard they weigh from 74 to 94 gm. and on the irradiated lard from 92 to 125 gm. These weighings were made 3 weeks after the beginning of the experiment on a ration carrying but 2 per cent of the fats.

The suggestions carried by the data presented in this paper are obvious. As we realize that we have already indulged extensively in unorthodox speculations we shall withhold further statement of probabilities, and what we believe to be facts, for future publication.

SUMMARY.

By irradiation with the quartz mercury vapor lamp, rat rations can be activated, making them growth-promoting and bone-calcifying, to the same degree as when the rats are irradiated directly. This activation takes place when the ration is irradiated in an open dish or in a stoppered Pyrex or quartz flask, filled with air or CO_2, but not in a brown glass bottle. The activation is not destroyed by subjecting the ration to a vacuum, heating it for 45 minutes at 96°C., or letting it stand for 24 hours at room temperature.

Confirming the observations of Goldblatt and Soames, liver taken from irradiated rats is growth-promoting while liver from non-irradiated rats is inactive. The same was found true of lung and muscle tissue. Inactive muscle, exposed, after removal from the body, to the radiations of the lamp was found to have become activated, being both growth-promoting and bone-calcifying. Liver treated the same way also promoted bone calcification. The activity of liver taken from irradiated rats was not destroyed by drying at 96° for 24 hours and then keeping it in the laboratory in a stoppered bottle for 2 months.

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ON THE MECHANISM OF PHLORHIZIN DIABETES.

SECOND PAPER.

By THOMAS P. NASH, Jr., and STANLEY R. BENEDICT.

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(Received for publication, July 7, 1924.)

In a recent publication (1) Ringer has given the results of the coincident administration of insulin and glucose to phlorhizinized dogs. Of the sugar thus fed he recovered appreciably smaller quantities than would be expected had glucose alone been administered. The discrepancy, he believes, represents a typical insulin effect upon carbohydrate combustion; in fact, Ringer proposes the use of phlorhizinized dogs in assaying insulin directly in terms of glucose utilized. These findings are taken to discredit the suggestion of Nash and Benedict (2) that phlorhizin effects an intrinsic impairment of the sugar-burning mechanism, and to be indicative, rather, "of a phlorhizin injury to the pancreas, preventing the production of the antidiabetic hormone."

The view of the mechanism of phlorhizin diabetes advanced by Nash and Benedict was necessarily tentative and speculative, but it was at the time, and we still believe it to be, essentially in accord with the established facts. It does not seem to us, as it does to Ringer, "unlikely that a gram of phlorhizin, administered daily for 3 days, would effectually blockade the vast frontier of sugar-metabolizing cells of a large dog." Assuming that the 24 hour energy requirement of a 10 kilo dog is 700 calories, of which three-fourths, or 525 calories, are supplied by glucose, it may be estimated that the total weight of glucose burned during the period is 131 gm. This is a rate of 5.4 gm. of glucose consumed per hour, or about 0.1 gm. of glucose per minute. Thus for any short period of metabolism the amount of glucose burned is exceedingly small, and there is no apparent reason

that small amounts of phlorhizin (even less than 1 gm.) should not "saturate" the glucose receptors provided the combination is stable.

A theory of specific phlorhizin injury to the pancreas, on the contrary, can scarcely be reconciled with several important objections. No histological change is detected in the pancreas of the phlorhizinized animal (3). The historic experiment of Zuntz (4), who injected phlorhizin into one renal artery and obtained sugar first from the kidney of that side, is fundamental in its bearing upon the issue. It is well known, also, that after complete ablation of the pancreas the highest D:N ratio obtained is about 2.8, whereas Minkowski, Hédon (5), and others have shown that phlorhizin has practically the same effect upon a depancreatized dog as upon an otherwise normal dog, increasing the glycuresis and lowering the blood sugar.

These facts raise a strong presumption against the validity of the theory proposed by Ringer. However, it is possible to apply to the theory the more direct and critical test of extracting, for insulin, the pancreas of the phlorhizinized dog. We have applied this test and have obtained insulin from the phlorhizinized dog pancreas.

General Procedure.

Fasting female dogs were used. The phlorhizin technique and analytical methods were those employed in our original experiments (2). The dogs were killed by bleeding from the femoral artery under local anesthesia, with a brief chloroform inhalation at the end. The pancreas was removed immediately after death, trimmed as free as possible of fat (in no case was there a considerable amount of fat), ground to a pulp with sand and a little acid alcohol in a mortar, and placed in the first alcohol acid extractive within 10 to 15 minutes. In our earlier experiments the procedure employed in extracting the pancreatic material was essentially that described by Doisy, Somogyi, and Shaffer (6), but later we incorporated the modifications recommended by Fenger and Wilson (7).

The potency of the pancreatic extracts thus prepared was tested in the usual way upon rabbits, which were fasted for 18 to 20 hours before the test. Blood was taken from the marginal

ear veins before, and 2 hours after, the injection, and the blood sugar determined by the Folin-Wu method. The rabbits were under observation for 5 hours following injection, and when convulsions occurred glucose solution was given subcutaneously. In every instance of convulsions the rabbit recovered completely and took offered food within 10 to 15 minutes after injecting 1 to 1.5 gm. of glucose in 10 per cent solution.

EXPERIMENTAL.

Data of the experiments are given in Tables I and II, the pancreatic extracts being numbered to correspond to the phlorhizinized dogs from which they were obtained.

In only two dogs, Nos. 5 and 6, are the D:N ratios open to the criticism that the phlorhizin effect was possibly not maximal. Dog 5 was pregnant, and after death was discovered to have been near term. The ratios for this dog were exceptionally constant throughout the 3 day phlorhizin period. Thus, in the first 24 hour period the total urine nitrogen was 5.45 gm., and the total urine sugar was 9.48 gm., the ratio being 1.74; for the second 24 hour period the corresponding values were 6.86 and 13.89 gm., and the ratio 2.02; the urine of the third 24 hour period was collected in four fractions whose respective ratios were 2.15, 2.06, 1.99, and 2.11. It is an interesting speculation whether the fetuses were not phlorhizinized and consequently withdrew from the blood of the mother sugar that otherwise would have been excreted. It may be noted that the blood sugar of the mother was at the low level of 47 mg. per 100 cc., whereas the sugar content of the amniotic fluid (by Benedict's acetone method for sugar in normal urine) was 75 mg. per 100 cc. In any event, this dog had received a quantity of phlorhizin which experience has shown to be sufficient to produce the maximal effect.

Dog 6 was a very large collie, and a most extraordinary animal in behavior. It was kept caged in the laboratory for a week prior to experimentation, and sulked throughout the whole time, refusing food and practically all water. Urine taken by catheter just before the first phlorhizin dosage showed no albumin or casts, but solidified with urea nitrate crystals when treated with nitric acid. The animal was used only because of the promise of an exceptionally large pancreas, and the possibility that phlor-

TABLE I.

Data of 24 Hour Periods in the Phlorhizinized Dogs.

Phlorhizin injection begun on the 3rd fasting day.

Date.	Urine.			Blood sugar.	Remarks.
	Total N.	Total sugar.	D:N		
Dog 5. Weight, 11.2 kg. Beginning Mar. 8, injected subcutaneously 1 gm. of phlorhizin in olive oil, daily.					
1924	*gm.*	*gm.*		*mg. per 100 cc.*	
Mar. 11	7.93	16.68	2.10	47	Dog killed Mar. 11. Pregnant, near term.
Dog 6. Weight, 22.2 kg. Phlorhizin in olive oil injected subcutaneously as follows: 1 gm. at 9.00 a.m., Mar. 17; 1 gm. at 5.30 p.m., Mar. 17; 1.5 gm. at 9.00 a.m., Mar. 18.					
Mar. 19	11.12	34.69	3.12	45	Dog killed Mar. 19.
Dog 7. Weight, 12.0 kg. Beginning Mar. 29, injected subcutaneously 1 gm. of phlorhizin in olive oil, daily.					
Apr. 1	10.59	36.78	3.47	58	Dog killed Apr. 1.
Dog 8. Weight, 10.8 kg. Beginning Apr. 12, injected subcutaneously 1 gm. of phlorhizin in olive oil, daily.					
Apr. 15	9.39	33.18	3.53	68	
" 18	7.60	25.70	3.38	60	Dog killed Apr. 18.
Dog 9. Weight, 11.3 kg. Beginning May 9, injected subcutaneously 1 gm. of phlorhizin in olive oil, daily.					
May 12	8.06	27.39	3.40	77	
" 17	5.99	23.88	3.98		Dog killed May 17.
Dog 10. Weight, 7.8 kg. Beginning May 6, injected subcutaneously 1 gm. of phlorhizin in olive oil, daily.					
May 9	8.08	26.60	3.29		
" 10	7.64	27.23	3.56		Dog killed May 10.

hizin would produce its normal effects. However, the animal continued to take water only very sparingly, and by March 19, when a total of 3.5 gm. of phlorhizin had been given, was in such poor condition that it was killed and the pancreas processed.

With the exception of these two dogs the animals of our series were entirely normal in condition and behavior. Dog 8 received

TABLE II.

Summary of Data of the Rabbit Test for Insulin in the Extracts of the Pancreas of Phlorhizinized Dogs.

Pancreatic extract No.	Weight of pancreas.	Total volume of "final extract."	Volume of "final extract" injected.	Weight of rabbit.	Blood sugar.		Remarks.
					Before injection.	2 hrs. after injection.	
	gm.	*cc.*	*cc.*	*kg.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
5	17.5	5.5	1.0	1.6	135	72	No convulsions in 2 hrs.
6	26.5	7.0	1.5	2.1	115	70	" " " 2 "
7	22.5	5.5	1.0	1.4	125	54	Convulsions 2 hrs. 50 min. after injection. Antidoted by glucose.
8	19.0	5.5	1.5	1.6	150	81	No convulsions in 5 hrs.
9	14.0	4.5	1.0	2.2	120	49	Convulsions 2 hrs. after injection. Antidoted by glucose.
9			3.4	2.3	98	57	Convulsions 2 hrs. 35 min. after injection. Antidoted by glucose.
10	14.0	5.5	1.5	1.4	100	66	No convulsions in 5 hrs.
10			3.5	2.2	135	77	" " " 5 "
10-2*		2.0	2.0	1.4	120	52	Convulsions 2 hrs. 15 min. after injection. Antidoted by glucose.

* This preparation was made from a second precipitate obtained by readjusting the pH of the alcohol decanted in the final stages of preparation of Lot 10.

daily for 6 successive days 1 gm. of phlorhizin, and Dog 9 was similarly phlorhizinized for 8 days; yet notwithstanding this prolonged phlorhizin effect the pancreatic extracts obtained were among the most potent we have prepared.

The data of Table II require little explanation. In the instance of pancreatic extract No. 10–2, this represents a second

yield from the alcoholic mother liquor in which preparation No. 10 was precipitated. When extract No. 10 failed to induce convulsions it was suspected that the critical hydrogen ion concentration for the precipitation of insulin had not been attained in the first manipulation. By more accurately adjusting the pH of the decanted alcohol to 5.5 a second precipitate was obtained, very small in amount and with more color than the first, but quite potent as shown in the table. It is probable that in some of our earlier and less potent extracts the reaction was not accurately enough controlled to give the maximal yield of insulin.

SUMMARY.

There are several well known facts which are not in harmony with Ringer's view that phlorhizin injures the pancreas, preventing the production of the antidiabetic hormone. Some of these facts have been pointed out, and the isolation of insulin from the pancreas of phlorhizinized dogs has been reported. The conclusion is therefore reached that Ringer's view of the mechanism of phlorhizin action is not tenable.

Addendum.—We had contemplated incorporating in the present paper a discussion of Ringer's experiments upon which is based the claim that insulin enables the phlorhizinized dog to burn glucose. A critical review by Colwell (Colwell, A. R., *J. Biol. Chem.*, 1924, lxi, 289) of Ringer's interpretation of his results has recently come to the attention of one of us. Colwell reaches the conclusion that Ringer's experiments fail to demonstrate sugar combustion. Since Colwell's communication will have appeared prior to the publication of the present paper, and since his criticism is essentially along the lines we should have adopted, we will simply state here that we also feel that Ringer failed to demonstrate that the phlorhizinized dog burns glucose after receiving glucose and insulin.

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THE CONCENTRATION OF VITAMIN B.

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Many efforts to concentrate the crude vitamins have undoubtedly been made, though few successful experiments are reported. As far as the present writers can judge, the work on the purification of the crude vitamins followed two distinctive principles. One aimed to find a specific reagent for precipitation or for extraction, the other to remove gradually the inert materials and thus progressively increase the potency of the material. We are inclined to believe that thus far the second procedure has led to more satisfactory results. The outstanding contribution in this direction is the work of Osborne and Wakeman,[1] who removed much of the inert material from yeast. Their vitamin fraction contained in concentrated form practically the entire mass of vitamin originally present in the yeast. A given weight of this material was ten times as potent as the same weight of dry yeast. A further progress towards concentration of vitamins was made by Seidell,[2] who with Lloyd's reagent adsorbed some of the active material contained in the Osborne and Wakeman fraction. Very recently, Seidell[3] reported still further progress. The material obtained by adsorption was further fractionated by means of picric acid. It is unfortunate that Seidell changed the technique for testing the potency of vitamin B and employed pigeons as the test animal instead of young rats used by Osborne and Wakeman. The results of Seidell cannot, therefore, be compared, in a quantitative sense, with those of Osborne and Wakeman.

[1] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.
[2] Seidell, A., *Pub. Health Rep.*, 1916, xxxi, 364.
[3] Seidell, A., *Pub. Health Rep.*, 1924, xxxix, 294.

The plan of our work was in a general way similar to that of Osborne and Wakeman inasmuch as we aimed to remove gradually the inert material and thus to increase the potency of the active fraction.

The starting point of our work was the fraction of Osborne and Wakeman. From this fraction some inert material could be removed by means of methyl alcohol. The part precipitated by this reagent was practically inactive. The active fraction in the concentrated filtrate could be again precipitated by the addition of 4 volumes of ethyl alcohol. The material prepared in this manner was a dry powder, but seldom more than twice as potent as the original material.

In the early experiments, this material was used for further concentration of vitamin B. Later, the original Osborne fraction was used. The further purification was accomplished by means of various adsorbents.

Lloyd's Reagent Process.

Inasmuch as Lloyd's reagent had already been used successfully by Seidell, this reagent was the first to be tested. The surface of this reagent acts as a salt of a weak acid, and the reaction is a simple chemical reaction of double exchange only slightly complicated. It is, therefore, natural to expect that the "adsorbing power" should be affected, on the one hand, by the degree of ionization of the active surface of the adsorbent, and, on the other hand, by the degree of dissociation of the substance to be adsorbed. Since both depend on the hydrogen ion concentration of the medium, it is natural to expect that for each substance there must exist a certain hydrogen ion concentration when the adsorption is optimal. Hence, the adsorbing power of Lloyd's reagent was tested at different hydrogen ion concentrations. As may be seen from the curve, the optimal adsorption is at a pH of 4 (Fig. 1). On the other hand, the optimal extraction is at a pH of 9. The potency of this material was from two to three times as high as that of the original material.

Barium Hydroxide Process.

In the early experiments with Lloyd's reagent the procedure of Seidell was followed and the active material was extracted from

the adsorbent by means of an aqueous solution of barium hydroxide. Comparatively little of the active material was extracted, whereas, by means of sodium hydroxide, the extraction was complete. The idea suggested itself that barium ions formed an insoluble complex with the active principle. It was therefore attempted to precipitate the vitamin from the solution by means of a solution of barium hydroxide. The precipitation was not complete and the filtrate still contained an appreciable amount of active material. The maximum potency of the precipitate was about eight times that of the original material.

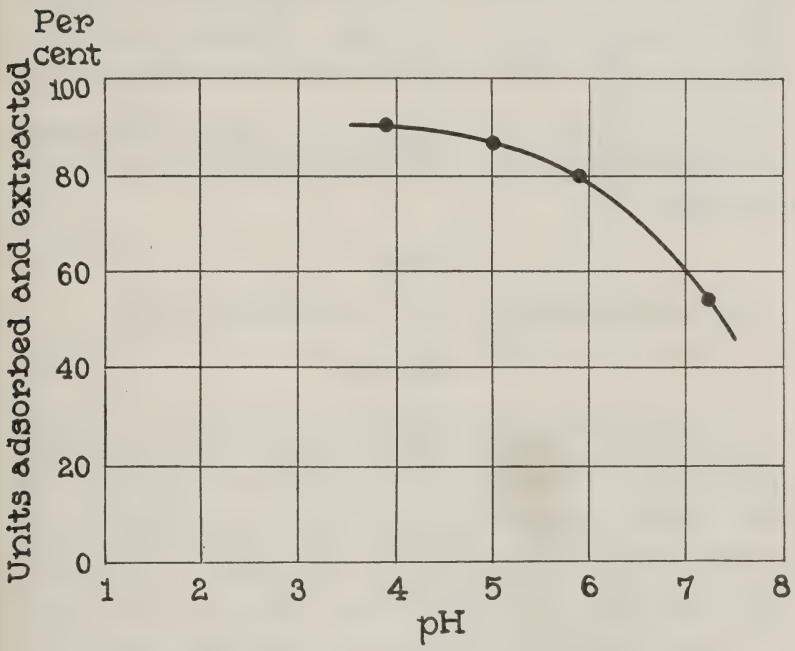

FIG. 1. Adsorption by Lloyd's reagent (Experiments 44 to 47).

In the early days of work on enzymes, calcium hydroxide was employed for precipitation of pepsin. However, soluble phosphates were added preliminary to the precipitation and the calcium phosphate thus formed, adsorbed the enzyme. It was natural to think that the vitamin was also adsorbed by barium phosphate, since soluble phosphates are present in the crude active material. Besides phosphoric acid the crude material contains other substances capable of forming insoluble barium salts. No-

table among these is nucleic acid. Since the precipitation of the vitamin by means of barium hydroxide was incomplete, it was logical to assume that the quantity of vitamin removed from the solution was proportional to the quantity of insoluble barium salt formed. It was expected that addition of soluble phosphates or of sodium nucleate would increase the quantity of active material brought down by the barium precipitate. Addition of phosphoric acid or of nucleic acid to the filtrate obtained from the first precipitation, however, did not bring down any of the vitamin contained in the filtrate. When they were added to the original solution preliminary to treatment with barium hydroxide, the yield of adsorbed vitamin remained unchanged. Apparently the vitamin is precipitated by a barium salt of an acid other than phosphoric or nucleic acid.

Attempts to increase the potency by repeated precipitation of the liberated vitamin with barium hydroxide were also unsuccessful.

Kaolin.

This was tested but gave rather unsatisfactory results.

Silica Gel.

This reagent was found to be the most satisfactory. The most active material was obtained when adsorption was carried out at pH 5 (Fig. 2). Furthermore, it was found that from this gel the active material could be reextracted not only by alkalies but also by acids at pH 3. This peculiarity of the silica-vitamin system seemed of considerable importance, inasmuch as vitamin B is much more stable in acid than in alkali. It proved, in the course of the work, that at pH 3 only part of the active material is extracted and that a larger proportion and a more potent material can be extracted by a subsequent extraction at pH 9.

Further Purification by Repeating the Adsorption with a Second Adsorbent.

This method proved to a certain degree successful. Comparatively little was accomplished when the material obtained by the barium process was readsorbed by Lloyd's reagent and *vice versa*,

but considerable increase in potency was obtained when the solution containing the material obtained by the barium process was treated with silica gel. The extract at pH 9 of the silica material was very frequently active in quantities of 0.0001 gm. and not infrequently in quantities of 0.00005 gm. of nitrogen per day. Thus this material is between 200 to 400 times as potent as dried yeast. It must be mentioned, however, that this degree of purity is obtained only with considerable loss of material. Only a fraction of 1 per cent of the original number of units is obtained as extremely potent material.

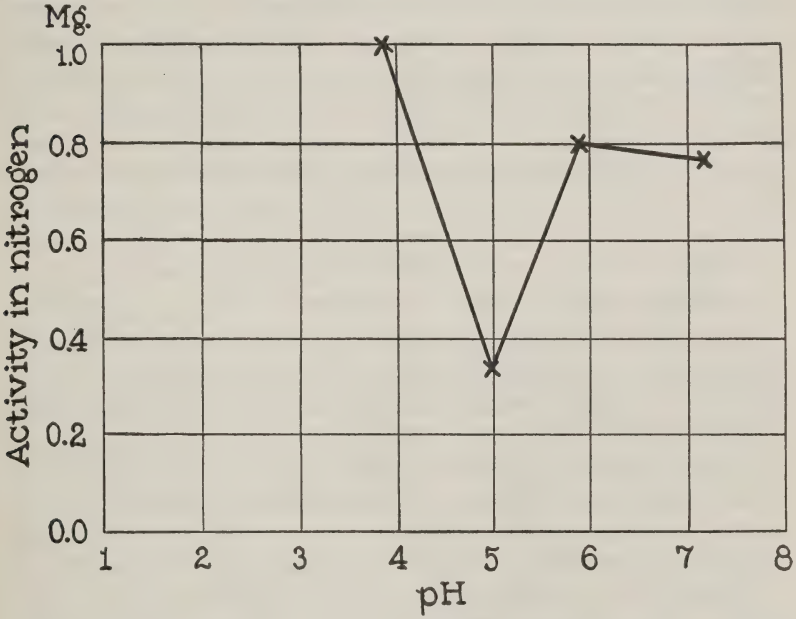

Fig. 2. Adsorption by silica gel (Experiments 70 to 73).

A more convenient method for improving the potency of the material prepared by the barium process is the following. The original barium precipitate is suspended in water through which a stream of carbonic acid gas is passed. The principle underlying this method is the following. In the barium precipitate, the substances with acidic properties stronger than carbonic acid remain undissociated and stay in the solid phase, whereas the substances with the opposite properties pass into solution. *A*

priori, it was not possible to predict in which of the fractions the active material would be found. Experience has shown that by this process a considerable part of the inert material passed into solution, whereas the potent material is contained in the insoluble part. When the latter is freed from barium the material is active in about 0.00017 gm. of N per day. This material is obtained with very little loss of vitamin and, therefore, will serve as starting material for our further efforts to concentrate the growth-promoting principle contained in yeast.

In conclusion, a word may be said in regard to two important contributions in the field of vitamin research. One is by Seidell on a crystalline picrate of vitamin B.[3] According to Seidell, the elementary composition of the material is $C_6H_{18}O_2N_3OHC_6H_2(NO_2)_3$, which makes the nitrogen of the potent part about 10 per cent of the picrate. Thus, the prophylactic dose for young pigeons is the equivalent of 0.0002 gm. of nitrogen every 2nd day. The standards of testing employed by Seidell and by us are so different that it is difficult to compare the results of the two investigations. It seems unlikely that the picrate of Seidell and our concentrate have potencies of different orders of magnitude. Yet we are certain that our material is not an individual substance, but a complex mixture. The pure vitamin, if ever isolated, will prove many times more potent than the picrate of Seidell or than our present concentrate.

A word may be also said in regard to the crystalline "bios" of Eddy, Kerr, and Williams.[4] Their substance evidently was prepared in small quantities so that as yet its elementary composition is not quite certain. As thus far given, it is the following. C = 43.29, H = 8.31, N = 25 per cent (approximately), m. p. = 223°C. The chemical properties of the substance are not given. Many years ago, J. A. Mandel and E. K. Dunham prepared from a commercial product, zymase, a very interesting substance, an adenine hexoside. From private communications we know that these authors failed to isolate the same substance from yeast. Recently one of us (Levene) prepared a considerable quantity of it from yeast. The composition is C = 44.41, H = 5.09, N =

[4] Eddy, W. H., Kerr, R. W., and Williams, R. R., *Proc. Soc. Exp. Biol. and Med.*, 1923-24, xxi, 307.

23.57, m.p. = 214°C. These analytical data, except for the hydrogen, remind one of those given by Eddy, Kerr, and Williams. At one time Williams and Seidell[5] suggested that vitamin B may be a tautomeric form of adenine. Therefore, the substance was tested for its growth-promoting properties. The result was negative. When the publication of Eddy, Kerr, and Williams appeared, the resemblance of the adenine hexoside to the crystalline "bios" naturally aroused our curiosity as to the possible "bios" effect of the adenine hexoside. Dr. Dubin, of the H. A. Metz laboratories, kindly performed the test and reported negative results. It is somewhat unfortunate that Eddy, Kerr, and Williams did not report on the chemical properties of their substance. It is possible that the resemblance in elementary composition is only accidental and that the "bios" of Eddy, Kerr, and Williams has no relationship with the adenine hexoside. Of course, there is a marked discrepancy in the hydrogen value of the two substances.

EXPERIMENTAL.

The biological effect of vitamins on the growth of young rats was used for estimating the vitamin content in the various extracts. Rats about 6 weeks old were used for this purpose after having been fed on the "basal diet" (McCollum) for 3 weeks. The average weight of the animals was 50 gm.; during the week immediately preceding the experiment, they lost on the average of about 5 to 10 gm. in weight. The substances were given in solution. These were carefully neutralized and preserved by the addition of 30 per cent alcohol. After 3 or 4 days, the animals were again weighed and the change in weight gave an indication of the vitamin content of the solution. The quantity of solution given to the animals was determined by its nitrogen content.

For the sake of convenience, the following arbitrary way of standardizing the potency of the material was adopted.

From "normal growth" curves of several rats the average increase in weight during 3 days for a body weight of 40 to 70 gm. is found (5 to 7 gm.) Considering this increase normal, the amount of the substance (measured by the quantity of nitrogen)

[5] Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.

was determined which would be required to give a normal increase in weight in the test animals during the feeding period. It is realized that the adopted unit may be insufficient to bring about a normal course of the entire period of growth up to maturity. However, it offers a rapid and reliable method for standardizing the relative strength of various products.

Starting Materials.

Substances were used which had been prepared either exactly according to the directions of Osborne and Wakeman or by a method identical with that in principle, but in which the details were slightly modified. Attempts to use other extracts prepared in different ways were not very successful. The potency of the fraction of Osborne and Wakeman differed considerably depending upon the character of the yeast. At a time when brewers' yeast was not accessible, bakers' yeast was employed. It was found that different samples of bakers' yeast differed in their vitamin content. The potency of the materials was approximately the following.

From Brewers' Yeast.

No. 4. A sample prepared exactly according to the directions of Osborne and Wakeman, potency 0.003 gm.

No. 223. An extract of brewers' yeast prepared by boiling 100.0 gm. of yeast with 500 cc. of water to which 1 cc. of acetic acid was added, potency 0.003 gm. Occasionally, samples prepared by the Osborne and Wakeman process had a potency of 0.002 gm. These samples were equal in potency to the material prepared by Osborne and Wakeman.

From Bakers' Yeast.

No. 2. A sample prepared according to Osborne and Wakeman, potency 0.0025 gm.

No. 3. A sample prepared by Osborne and Wakeman process and purified by the methyl alcohol process.

Adsorption by Lloyd's Reagent.

Adsorption experiments with Lloyd's reagent were made at different H ion concentrations, determined colorimetrically by the drop method. This pH was kept constant during the adsorption period. Per gram of nitrogen about 25 gm. of Lloyd's reagent were used. The adsorption was completed after continuous shaking for 2 hours at room temperature. The adsorbed material

was extracted from the Lloyd's reagent by a dilute alkaline solution. To each 20 gm. of Lloyd's reagent, 1 liter of solution with a pH of 9 to 10 was used; *i.e.*, about 0.5 per cent NaOH. The extraction was practically complete after one treatment.

The results can be seen in Fig. 1. At pH 4 to 5 the adsorption is practically complete (90 per cent); at higher values of pH, it is lowered.

Nearly all extracts have the same activity (1.1 to 1.2), regardless of the strength of the original solution. Extractions of Lloyd's reagent in alcoholic solution resulted in material with a higher potency (0.6) (Experiment 54). This result is more promising and indicates that by extracting Lloyd's reagent in a slightly alkaline medium (pH 9.0) with the addition of alcohol, the product can be improved.

Experiments with Lloyd's Reagent.

1. At different values of pH (Nos. 44 to 47).
2. At pH 5—on a large scale (Nos. 48 to 49).
3. With the addition of alcohol to the alkaline extraction (No. 54).

(44) No. 223.

(3)* |300| units 0.90 gm. N

pH 3.9

Filtrate No. 427 (inactive) 0.32 gm. N = |0| units

Extract No. 428 (1.2) 0.33 gm. N = |270| units

(45) No. 223.

(3) |500| units 1.50 gm. N

pH 5

Filtrate No. 419 (inactive) 0.35 gm. N = |0| units

Extract No. 420 (1.1) 0.28 gm. N = |250| units

* (_) means mg. of nitrogen per unit potency.

(46) No. 223.

(3) |300| units 0.90 gm. N

pH 5.9

Filtrate No. 421	Extract No. 422
(inactive) 0.38 gm. N	(1.1) 0.26 gm. N
= |0| units	= |240| units

(47) No. 223.

(3) |300| units 0.90 gm. N

pH 7.2

Filtrate No. 429	Extract No. 430
(inactive) 0.43 gm. N	(1.3) 0.23 gm. N
= |0| units	= |170| units

(48) No. 4.

(3) |±1,600| units

pH 5

Filtrate No. 448	Extract No. 449
(inactive) 2.9 gm. N	(1.1) 1.56 gm. N
= |0| units	= |1,400| units

(49) No. 223.

(3) |1,800| units

pH 5

Filtrate No. 444	Extract No. 445
(inactive) 3.5 gm. N	(1.3) 1.67 gm. N
= |0| units	= |1,300| units

(54) No. 505.

(1.0) |1,100| units 1.1 gm. N

pH 5

Filtrate No. 510	Extract No. 511
(2.5) 0.60 gm. N	(0.6) 0.27 gm. N
= |240| units	= |450| units

Adsorption by Kaolin.

These experiments (Nos. 64 to 67) were made in exactly the same way as the Lloyd's reagent experiments. The results, how-

ever, were not so consistent. The general trend is the same: 90 per cent of adsorption at pH 4 and 5; 50 per cent at pH 7.2. The extraction of the kaolin is not very easy because some aluminum hydroxide dissolves in the alkali and very likely adsorbs a great deal of the vitamins. Only in one case (pH 5.0) a fairly satisfactory extract with comparatively little loss was obtained. This adsorbent was abandoned.

Experiments with Kaolin.

(64) No. 450.

(1.8) |190| units 0.34 gm. N

pH 3.9

Filtrate No. 494	Extract No. 495
(13) 0.26 gm. N	(1.2) 0.05 gm. N
= |20| units	= |40| units

(65)

(1.8) |190| units 0.34 gm. N

pH 5.0

Filtrate No. 496	Extract No. 497
(16) 0.26 gm. N	(0.5) 0.06 gm. N
= |16| units	= |120| units

(66)

(1.8) |190| units 0.34 gm. N

pH 5.9

Filtrate No. 498	Extract No. 499
(6.6) 0.29 gm. N	(2.3) 0.04 gm. N
= |40| units	= |20| units

(67)

(1.8) |190| units 0.34 gm. N

pH 7.2

Filtrate No. 500	Extract No. 501
(3) 0.30 gm. N	(1.7) 0.04 gm. N
= |100| units	= |25| units

Precipitation with Barium Hydroxide.

As was stated in the introductory part, the suggestion to make use of barium hydroxide for precipitating the active principle came from the observation that aqueous barium hydroxide compared with sodium or potassium hydroxide of equivalent strength, extracted the active principle from Lloyd's reagent less perfectly. The procedure in employing the barium hydroxide process is illustrated by the following experiment.

To a portion of No. 223, prepared from 1 kilo of dry yeast, were added 2.5 liters of cold saturated barium hydroxide solution. The precipitate which formed was centrifugalized and then suspended in water and an excess of 10 per cent sulfuric acid was added. The mixture was shaken until all lumps were dissolved and the barium sulfate was removed by centrifugalization. Both filtrate and precipitate were tested. Materials 2 and 4 were treated in the same manner.

Experiments with Barium Hydroxide.

(11) No. 4.

(3.0) |5,130| units 15.4 gm. N

Filtrate No. 375	Precipitate No. 374
(10) 10.72 gm. N	(0.46) 1.34 gm. N
= |1,000| units	= |2,900| units

(12) No. 4.

(1.7) |5,000| units 8.5 gm. N

Filtrate No. 576	Precipitate No. 577
(4) 6.6 gm. N	(0.35) 1.0 gm. N
= |1,600| units	= |3,000| units

Adsorption by "Silica Gel."

The experiments with adsorption by silica gel were performed on material which had already been purified by the barium process, or by means of Lloyd's reagent.

Experiments with Silica Gel.

1. At different values of pH (Nos. 70 to 73).
2. Acid and alkaline extractions of silica gel (No. 74).
3. Fractional extraction of silica gel (Nos. 83 and 84).

(70) No. 439 (Ba process).

(1.4) |280| units 0.40 gm. N

pH 3.9

Filtrate No. 482
(3.3) 0.43 gm. N
= |130| units

Extract No. 483
(1.0) 0.007 gm. N
= |7| units

(71)

(1.4) |280| units 0.40 gm. N

pH 5.0

Filtrate No. 484
(inactive) 0.41 gm. N
= |0| units

Extract No. 485
(0.33) 0.005 gm. N
= |15| units

(72)

(1.4) |280| units 0.40 gm. N

pH 5.9

Filtrate No. 486
(inactive) 0.33 gm. N
= |0| units

Extract No. 487
(0.8) 0.021 gm. N
= |29| units

(73)

(1.4) |280| units 0.40 gm. N

pH 7.2

Filtrate No. 488
(9) 0.40 gm. N
= |44| units

Extract No. 489
(0.7) 0.021 gm. N
= |30| units

(74) No. 505 (Lloyd's extract).

(1.0) |1,600| units 1.60 gm. N

pH 5

Filtrate No. 506

(1.3) 1.41 gm. N
= |1,100| units

Silica in 3 parts

Silica plain
(0.20) 0.002 gm. N
= |10| units

Silica extracted
(pH 9.0) No. 508
(0.25) 0.006 gm. N
= |25| units

Silica extracted
(pH 3.0) No. 507
(0.22) 0.006 gm. N
= |30| units

(83) No. 545.

(1.5) |420| units 0.63 gm. N

Filtrate No. 557
(2.6) 0.59 gm. N
= |230| units

Silica extracted
I. Extract (pH 5)
(inactive) 0.008 gm. N
= |0| units

II. Extract (pH 3)
(0.75) 0.009 gm. N
= |12| units

III. Extract (pH 3)
(inactive) 0.0005 gm. N
= |0| units

IV. Extract (pH 9)
(0.06) 0.0005 gm. N
= |10| units

(84) No. 577 (Ba precipitate).

(0.35) |3,000| units 1.04 gm. N

Extracted 200 gm. silica
pH 5

Filtrate No. 583
(0.60) 0.89 gm. N
= |1,500| units

I. Extract (pH 3) No. 584
(0.25) 0.11 gm. N
= |440| units

II. Extract (pH 9) No. 585
(0.11) 0.007 gm. N
= |64| units

III. Extract (pH 9) No. 586
(0.10) 0.0013 gm. N
= |13| units

Purification of Barium Precipitate by Means of Carbonic Acid.

The precipitate, prepared in the usual way, was suspended in water and carbonic acid gas was passed through it for 5 hours. The reaction product was centrifugalized. The precipitate and the filtrate were freed from barium by means of sulfuric acid. Each fraction was neutralized and concentrated. The results of the experiment are given on the following diagram.

Barium Precipitate with CO_2.

No. 4.

(1.7) |2,300| units 4.0 gm. N

Filtrate No. 587

(6.6) 3.5 gm. N
= |500| units

Precipitate + CO_2

Filtrate No. 589
(3.3) 0.37 gm. N
= |100| units

Residue No. 590
(0.17) 0.30 gm. N
= |1,500| units

THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF PEPTIDES.

I. ON THE SYNTHESIS, ON THE PHYSICAL CONSTANTS, AND ON THE RATES OF HYDROLYSIS OF METHYLATED PEPTIDES.

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It has been known for a long time that the protein molecule is a poly-aminoacid. The information that the units are linked in the form of amides is an acquisition of recent years. The conception was formulated on purely speculative grounds by Hofmeister and demonstrated convincingly by the synthetic work of Fischer and his collaborators. The great activity in the field of physical chemistry of proteins was stimulated and made possible by the knowledge of the mode of linking which Fischer termed peptide linking.

In the future, as in the past, progress will depend upon further achievements in the synthesis of peptides. The ingenious process of Fischer for the synthesis of peptides consisted in combining a halogen-substituted acid chloride with an amino acid and in converting this derivative of the amino acid into a peptide in the following manner.

$$\mathrm{R{\cdot}CHCl{\cdot}COCl + R{\cdot}CHNH_2{\cdot}COOH \rightarrow R{\cdot}CHCl{\cdot}CO{\cdot}NH{\cdot}\overset{\displaystyle R}{\overset{|}{C}}H{\cdot}COOH \rightarrow}$$

$$\mathrm{R{\cdot}CHNH_2{\cdot}CO{\cdot}NH{\cdot}\overset{\displaystyle R}{\overset{|}{C}}H{\cdot}COOH}$$

For the synthesis of peptides of the simple amino acids, the procedure gave splendid results, but a general method is still lacking for the synthesis of peptides of the complex amino acids such as arginine, histidine, lysine, etc. The chief difficulty lies

in the absence of a suitable method for protecting the amino groups (primary or secondary). The long list of synthetic peptides prepared by Fischer and his collaborators and later by Curtius and his collaborators contains practically none of the complex amino acids. *A priori*, it seemed that the polarity of an amino acid should not be much affected by the methylation of the amino group, and that, therefore, the general properties of the methylated peptides should resemble very closely those of the simple peptides not only in a qualitative but also in a quantitative sense. However, before engaging in the preparation of a large number of synthetic methylated peptides, it was deemed advisable to test the properties of the following group of dipeptides: (1) glycyl-glycine, (2) sarcosyl-glycine, (3) glycyl-sarcosine, and finally of (4) sarcosyl-sarcosine. These peptides were analyzed, on the one hand, with regard to their constants of dissociation, and on the other, in regard to their resistance against hydrolytic agents, as it was realized that a certain connection ought to exist between these two properties.

Table I contains the results of the measurement of the dissociation constants of the two amino acids, glycine and sarcosine, and of the four peptides enumerated above.

The constants in Columns 5 and 6 are calculated on the assumption that pK_1 is the acidic constant and that $pK_2 = pK_w - pK_b$ represents the dissociation of the amino group.[1,2]

All the constants listed above agree well with the values expected from the constitution on the basis of simple laws which will be discussed more fully in a future publication.

The results of the measurements of the rate of hydrolysis are summarized in Table II. The details of the procedure are given in the experimental part. Here it suffices to mention that in the principal experiments, the active concentration of the hydrogen ion was practically equimolecular with the concentration of unhydrolyzed peptide throughout the hydrolysis. Under these conditions, the hydrolysis followed the bimolecular course. The reaction proceeded normally in the case of all peptides except sarcosyl-sarcosine, in which case the binding power of the reaction product for acid was decreased early in the experiment. Later,

[1] Adams, E. Q., *J. Am. Chem. Soc.*, 1916, xxxviii, 1503.
[2] Bjerrum, N., *Z. physik. Chem.*, 1923, civ, 147.

the binding power for acid rose and, towards the end of the experiment, the reaction followed practically a normal course. *A priori*, it was to be expected that the mono-methylated and the non-methylated peptides should also show a diminished binding

TABLE I.

Dissociation Constants and Isoelectric Points at 25.0°C. (Glycyl-Glycine Measured at 30°C.).

(1)	(2)	(3)	(4)	(5)	(6)
Substance.	pK_1	pK_2	Isoelectric point. pI	$K_a \times 10^3$	$K_b \times 10^5$
Glycine	2.33	9.64	5.98	4.68	7.24
Sarcosine	2.23	10.01	6.12	5.89	17.0
Glycyl-glycine (30°C.)	3.12	8.07	5.59	0.759	0.195
Sarcosyl-glycine	3.10	8.51	5.80	0.794	0.537
Glycyl-sarcosine	2.83	8.54	5.68	1.48	0.576
Sarcosyl-sarcosine	2.86	9.10	5.98	1.38	2.09

TABLE II.

Rates of Hydrolysis of Molar Solutions of Dipeptides with 1.0 Equivalent H Ion Concentration at 100°C.

(1)	(2)	(3)	(4)	(5)
Substance.	Calculated from pK values of glycine and sarcosine.		Found.	
	$x \cdot 10^{(8+pK_1-pK_2)}$	Relative values.	$k \times 100$	Relative values.
Glycyl-glycine	$4.90\,x$	100	21.4	100
Sarcosyl-glycine	$3.89\,x$	79	13.7	64
Glycyl-sarcosine	$2.09\,x$	43	12.3	57
Sarcosyl-sarcosine	$1.66\,x$	34	3.2	15

x is an unknown factor.

power for acids under proper conditions. These expectations were realized when a lower concentration of acid was used; namely, 1.3 equivalents of the peptide concentration. The assumption of the formation of an anhydride of the peptide is the simplest way of explaining the decrease in acid-binding power.

In the case of sarcosyl-sarcosine this reaction is not negligible, but reaches an equilibrium early in the experiment. Thus, the effective concentration of the peptide undergoing hydrolysis was determined as described in the experimental part.

The values in Columns 2 and 3 of Table II are calculated on the assumption that *the resistance to hydrolysis is proportional to the product of the dissociation constants of the groups involved in the linkage.* Comparison of Columns 3 and 5 shows that this is

TABLE III.

Rates of Hydrolysis of Ring Compounds (Anhydrides) of the Dipeptides.

(1)	(2)	(3)		(4)	(5)
Substance.	Calculated from pK values of the dipeptides.			Found from the extent of hydrolysis after 2 hrs.	
	$x \cdot 10^{(8+pK_1-pK_2)}$	Relative values.		$\frac{1}{0.27V_{1.0}-V_{0.3}}$	Relative values.
		a	*b*		
Glycyl-glycine.....	1,120 x	23,000	100	$\frac{1}{0.01}$	100
Sarcosyl-glycine....	389 x	7,900	35	$\frac{1}{0.04}$	25
Glycyl-sarcosine....	195 x	4,000	17.4	$\frac{1}{0.57}$	17.6
Sarcosyl-sarcosine..	57.6 x	1,200	5.1	$\frac{1}{1.95}$	5.1

x has the same value as in Table II.

qualitatively correct. The correspondence is as close as could be expected, since there are other factors involved.

In a general way, this relationship is analogous to that observed by Sudborough and Lloyd[3] on saponification of esters.

It is to be expected that the stability of anhydrides, if formed, should follow the same relationship. Table III shows that this is true.

[3] Sudborough, J. J., and Lloyd, L. L., *J. Chem. Soc.*, 1898, lxxiii, 81; 1899, lxxv, 467.

The values in Column 3 *a* of Table III are on the same basis as those in Column 3, Table II. However, the two sets of figures should not be directly compared since the mechanism in ring-splitting is not the same as in peptide-splitting. Values relative to glycyl-glycine are given in Column 3 *b*.

A measure of the rate of ring hydrolysis (or the reciprocal of the rate of ring formation) is given in Column 4. It is obtained from hydrolysis data after 2 hours with 0.3 equivalent H ion concentration. If $V_{1.0}$ is the value of $V - V_0$ after 2 hours, as calculated from the constant (k), then 0.27 $V_{1.0}$ is the value of $V - V_0$ to be expected after the same time with 0.27 as great a concentration of H ion, if no ring formation occurred. The difference between this and the value found should indicate the extent of ring formation or resistance to ring hydrolysis. The reciprocal is a measure of the rate of ring hydrolysis.

A comparison of Columns 3 *b* and 5 shows a remarkably close agreement which indicates that *the tendency toward the formation* (or resistance toward the hydrolysis) *of ring compounds* (anhydrides) *of the dipeptides is proportional to the product of the dissociation constants of the groups involved in the linkage.*

Work on the action of enzymes on the methylated peptides is now in progress. When the present work was completed, there appeared the publication of Taru Imai[4] on "The action of erepsin on methylated peptides." He did not observe a greater resistance of the methylated peptides. He was dealing, however, with betaines, substances different in character from those employed in the present investigation.

The amino acid and dipeptide concentrations were determined by direct titration in aqueous solution with normal sodium hydroxide, using tropeolin O as indicator. The end-point was sufficiently high to indicate complete titration of all the amino acids or peptides which were used. By using a molar concentration of peptide, the results were thoroughly satisfactory without the use of formaldehyde or of alcohol.

[4] Taru Imai, H. S., *Z. physiol. Chem.*, 1924, cxxxvi, 192.

EXPERIMENTAL.

A. Preparation of Chloroacetyl-Glycine.

24 gm. of glycine were dissolved in 320 cc. of N sodium hydroxide and treated alternately with 44 gm. of chloroacetyl chloride (1.2 equivalents) and 528 cc. of N sodium hydroxide under cooling. Each was added in 10 equal portions. The solution was then neutralized with 96 cc. of 5 N hydrochloric acid and concentrated to dryness under diminished pressure. The residue was extracted with about 300 cc. of warm acetone and after concentrating it to about 100 cc. it was taken up in an equal volume of chloroform. On cooling, the chloroacetyl-glycine crystallized. The yield was 34 gm. which corresponds to about 70 per cent of the theory. The chloroacetyl-glycine was purified by dissolving it in about 1,500 cc. of boiling ether and concentrating the solution to about 100 cc. from which it was obtained in good crystalline form. The substance is very soluble in acetone, slightly soluble in hot ether and hot chloroform, and insoluble in petroleum ether. It melts at 98–100°C. For analysis it was dried *in vacuo* over sulfuric acid.

0.2000 gm. substance required (Kjeldahl) 13.00 cc. 0.1 N HCl.

$C_4H_6O_3NCl$.	Calculated.	N 9.24.
	Found.	" 9.10.

B. Preparation of Chloroacetyl-Sarcosine.

28.8 gm. of sarcosine were dissolved in 320 cc. of N sodium hydroxide and treated alternately with 44 gm. of chloroacetyl chloride (1.2 equivalents) and 528 cc. of N sodium hydroxide under cooling. Each was added in 10 equal portions. The solution was then neutralized with 96 cc. of 5 N hydrochloric acid and concentrated to dryness under diminished pressure. The residue was extracted with about 300 cc. of warm acetone. The acetone extract was concentrated to a small volume and treated with a large excess of chloroform. On cooling, the chloroacetyl-sarcosine crystallized. It was purified by recrystallizing from about 150 cc. of chloroform. The yield was 27 gm. which corresponds to 50 per cent of the theory. The substance is very soluble in acetone and hot chloroform, slightly soluble in ether, and insoluble in petroleum ether. It melts at 95–98°C. For analysis it was dried *in vacuo* over H_2SO_4.

0.2000 gm. substance required (Kjeldahl) 12.10 cc. 0.1 N HCl.

$C_5H_8O_3NCl$.	Calculated.	N 8.56.
	Found.	" 8.47.

C. Preparation of Glycyl-Sarcosine.

5 gm. of chloroacetyl-sarcosine were allowed to stand for 24 hours at room temperature with 25 cc. of ammonium hydroxide (sp. gr. 0.90). The solution was then concentrated under diminished pressure to a thick syrup. The syrup was then heated on the water bath with about 50 cc. of absolute alcohol until crystallization took place. The yield was 3.1 gm. The

material was purified by dissolving it in a minimum amount of hot water and adding 5 parts of hot absolute alcohol. It melts at 200–201°C. For analysis it was dried at 100°C. *in vacuo* over sulfuric acid.

0.09992 gm. required (Kjeldahl) 13.55 cc. 0.1 N HCl.

$C_5H_{10}O_3N_2$.	Calculated.	N 19.18.
	Found.	" 19.11.

D. Preparation of Sarcosyl-Glycine.

5 gm. of chloroacetyl-glycine were allowed to stand 2 days at room temperature with 15 cc. of a 33 per cent aqueous solution of methylamine. The solution was then concentrated under diminished pressure to a thick syrup. The syrup was heated on the water bath with about 50 cc. of absolute alcohol until crystallization took place. The yield was 3.3 gm. The material was purified by dissolving it in a minimum amount of hot water and adding about 10 parts of absolute alcohol. An oily precipitate was obtained which crystallized on standing. It melts at 195–197°C. For analysis it was dried at 100°C. *in vacuo* over sulfuric acid.

0.1000 gm. required (Kjeldahl) 13.8 cc. 0.1 N HCl.

$C_5H_{10}O_3N_2$.	Calculated.	N 19.18.
	Found.	" 19.32.

E. Preparation of Sarcosyl-Sarcosine.

10 gm. of chloroacetyl-sarcosine were allowed to stand 24 hours at room temperature with 30 cc. of a 33 per cent aqueous solution of methylamine. The solution was then concentrated under diminished pressure to a thick syrup. Owing to the probable formation of a methylamine salt of sarcosyl-sarcosine, this syrup does not crystallize on heating with absolute alcohol. The following procedure was therefore used to isolate the sarcosyl-sarcosine. The syrup was taken up in water and the solution acidulated with sulfuric acid. The acidulated solution was then shaken with moist silver carbonate until all the chlorine was removed. The silver sulfate and silver chloride were filtered off and the silver was removed from the filtrate with hydrogen sulfide. In order to remove the remaining methylamine, the solution was treated with 22 gm. of barium hydroxide and concentrated to dryness under reduced pressure. Water was added and the solution again concentrated to dryness. This was repeated until no more methylamine was given off. The barium was removed quantitatively from the solution by means of sulfuric acid, and the filtrate concentrated under diminished pressure to a thick syrup. On treating with absolute alcohol the syrup crystallized. The yield was 5 gm. The material was purified by dissolving it in a minimum amount of hot water, adding 10 parts of absolute alcohol, and heating the solution on the water bath until crystallization took place. It melts at 180–185°C. For analysis it was dried *in vacuo* at 78°C. over sulfuric acid.

0.1000 gm. required (Kjeldahl) 12.45 cc. 0.1 N HCl.

$C_6H_{12}O_3N_2$.	Calculated.	N 17.50.
	Found.	" 17.43.

Hydrolysis Experiments.

In the following tables (Tables IV to XI) will be found data on the hydrolysis of the four dipeptides formed from glycine and sarcosine. In the first four tables (Tables IV to VII) the concentration of hydrochloric acid (2.14 equivalents) was such as to give throughout the experiment, an H ion activity approximately equal to the concentration of unhydrolyzed dipeptide. For such a condition the reaction should be bimolecular and follow the equation:

$$kt = \frac{x}{a(a-x)} \tag{1}$$

In terms of volume of normal NaOH used in titration:

$$kt = \frac{V - V_0}{(V_\infty - V_0) - (V - V_0)} \tag{2}$$

where V = volume at time t, V_0 is the volume at zero time, and V_∞ is the volume for theoretical complete titration ($= V_0 + 5$). Since 0.005 mol was used in each case, the equation,

$$kt = \frac{(V - V_0)}{5.0 - (V - V_0)} \tag{3}$$

was used for the first three dipeptides.

Application of this formula to sarcosyl-sarcosine leads to impossible values below 7 hours and gives values for $k \times 100$ at 12.5, 16, and 24 hours equal to 1.41, 1.76, and 1.87, respectively. In order to test whether a different value, $V_\infty - V_0'$, would give a constant k over a wider range of time, if substitued for $V_\infty - V_0$, we transformed equation (2) into

$$k = \frac{1}{t_1}\frac{(V_\infty - V_0') - (V_\infty - V_1)}{(V_\infty - V_1)} = \frac{1}{t_2}\frac{(V_\infty - V_0') - (V_\infty - V_2)}{(V_\infty - V_2)} \tag{4}$$

Solving for $(V_\infty - V_0')$:

$$V_\infty - V_0' = \frac{t_1 - t_2}{\dfrac{t_1}{(V_\infty - V_2)} - \dfrac{t_2}{(V_\infty - V_1)}} \tag{5}$$

TABLE IV.

Hydrolysis of Molar Glycyl-Glycine with 1.0 Equivalent of H Ion.

5 cc. of 2.14 M hydrochloric acid per 0.005 mol of substance.

(1)	(2)	(3)	(4)
t	V	$V - V_0$	$k \times 100$
hrs.	*cc. M NaOH*		
0	15.65	0	
2	17.04	1.39	19.3
3	17.50	1.85	19.6
4	17.95	2.30	21.3
6	18.45	2.80	21.2
22.5	19.70	4.05	19.0
0	15.55	0	
1	16.40	0.85	20.5
7	18.55	3.00	21.4
12	19.20	3.65	22.5
16	19.35	3.80	19.8
20	19.75	4.20	26.2
24	19.85	4.30	25.6
40	20.20	4.65	(33.2)
Total average			21.4

TABLE V.

Hydrolysis of Molar Sarcosyl-Glycine with 1.0 Equivalent of H Ion.

5 cc. of 2.14 M hydrochloric acid per 0.005 mol of substance.

(1)	(2)	(3)	(4)
t	V	$V - V_0$	$k \times 100$
hrs.	*cc. M NaOH*		
0	15.65	0	
1	16.25	0.60	13.6
2	16.70	1.05	13.3
3	17.15	1.50	14.3
4	17.35	1.70	12.9
6	17.80	2.15	12.6
12	18.85	3.20	14.8
16	19.15	3.50	14.6
40	19.85	4.20	13.1
Average			13.7

TABLE VI.

Hydrolysis of Molar Glycyl-Sarcosine with 1.0 Equivalent of H Ion.

5 cc. of 2.14 M hydrochloric acid per 0.005 mol of substance.

(1)	(2)	(3)	(4)
t	V	$V - V_0$	$k \times 100$
hrs.	*cc. M NaOH*		
0	15.60	0	
1	15.90	0.30	(6.4)
2	16.35	0.75	(8.8)
3	16.80	1.20	(10.5)
4	17.10	1.50	(10.7)
5	17.25	1.65	(9.9)
6	17.60	2.00	(11.1)
12	18.55	2.95	12.0
16	18.95	3.35	12.7
20	19.15	3.55	12.2
24	19.35	3.75	12.5
40	19.75	4.15	12.2
Average of last 5 values.....			12.3

TABLE VII.

Hydrolysis of Molar Sarcosyl-Sarcosine with 1.0 Equivalent of H Ion.

5 cc. of 2.14 M hydrochloric acid per 0.005 mol of substance.

(1)	(2)	(3)	(4)
t	V	$V - V_0'$ (where $V_0' = 14.40$).	$k \times 100$
hrs.	*cc. M NaOH*		
0	15.40	1.00	
1	14.70	0.30	(5.3)
3	14.80	0.40	(2.4)
4	15.05	0.65	(2.6)
5	15.20	0.80	3.1
7	15.40	1.00	2.9
16	16.50	2.10	3.4
		($V_0' = 14.60$)	
0	15.60	1.00	
0.5	14.95	0.35	(12.4)
1	14.85	0.25	(4.3)
2	14.95	0.35	(3.1)
5	15.30	0.70	(2.6)
12.5	16.35	1.75	3.3
24	17.15	2.55	3.1
Average of best values after 5 hrs.....			3.2

TABLE VIII.

Hydrolysis of Molar Glycyl-Glycine with about 0.3 Equivalent of H Ion.

5 cc. of 1.3 M hydrochloric acid per 0.005 mol of substance.

(1)	(2)	(3)
t	V	$V - V_0$
hrs.	*cc. M NaOH*	
0	11.45	0
1	11.65	0.20
2	11.85	0.40
5	12.30	0.85
16	12.88	1.43
24	12.95	1.50
40	13.30	1.85

TABLE IX.

Hydrolysis of Molar Sarcosyl-Glycine with about 0.3 Equivalent of H Ion.

5 cc. of 1.3 M hydrochloric acid per 0.005 mol of substance.

(1)	(2)	(3)
t	V	$V - V_0$
hrs.	*cc. M NaOH*	
0	11.70	0
1	11.80	0.10
2	11.95	0.25
5	12.15	0.45
16	12.68	0.98
24	12.90	1.20
40	13.30	1.60

TABLE X.

Hydrolysis of Molar Glycyl-Sarcosine with about 0.3 Equivalent of H Ion.

5 cc. of 1.3 M hydrochloric acid per 0.005 mol of substance.

(1)	(2)	(3)
t	V	$V - V_0$
hrs.	*cc. M NaOH*	
0	11.70	0
1	11.40	−0.30
2	11.50	−0.20
3	11.85	+0.15
4	12.00	+0.30
6	12.17	+0.47
16	12.68	+0.98
24	12.78	+1.08
40	13.10	+1.40

TABLE XI.

Hydrolysis of Molar Sarcosyl-Sarcosine with about 0.3 Equivalent of H Ion.

5 cc. of 1.3 M hydrochloric acid per 0.005 mol of substance.

(1)	(2)	(3)
t	V	$V - V_0$
hrs.	*cc. M NaOH*	
0	11.55	0
1	10.20	−1.35
2	9.70	−1.85
3	9.75	−1.80
4	9.85	−1.70
7	9.95	−1.60
16	10.30	−1.25
24	10.70	−0.85
40	11.35	−0.20

From any two points the value of $V_\infty - V_0'$ may be calculated. The average value obtained from all the combinations from 5 to 24 hours time, was 6.0 cc. and this was found to hold throughout the whole range of the experiment. Hence, we may calculate the constant from the formula:

$$(6) \qquad kt = \frac{(V - V_0')}{6.0 - (V - V_0')} \quad \text{where } V_0' = V_0 + 5.0 - 6.0 = V_0 - 1.0$$

Owing to the anomalous behavior of sarcosyl-sarcosine a series of experiments (Tables VIII to XI) was run with only about 0.3 equivalent of H ion in order to see whether, by hydrolyzing at a lower rate, we could obtain evidence of ring formation in the other dipeptides. The lower curves of Figs. 1 to 4 show that there is a tendency toward an initial drop in all cases, but that this is most marked when the product of the dissociation constants of the dipeptide is greatest, thereby favoring more stable ring formation.

In each hydrolysis experiment, 0.005 mol of dipeptide (allowing for moisture) was weighed out and placed in a large test-tube with 5.00 cc. of the hydrochloric acid. The tube was sealed and placed in a water bath at 100°C. for a period of time indicated in the tables under Column 1. To determine the degree of hydrolysis

the tube was opened and the solution titrated directly in the tube with normal sodium hydroxide, using tropeolin O as indicator.

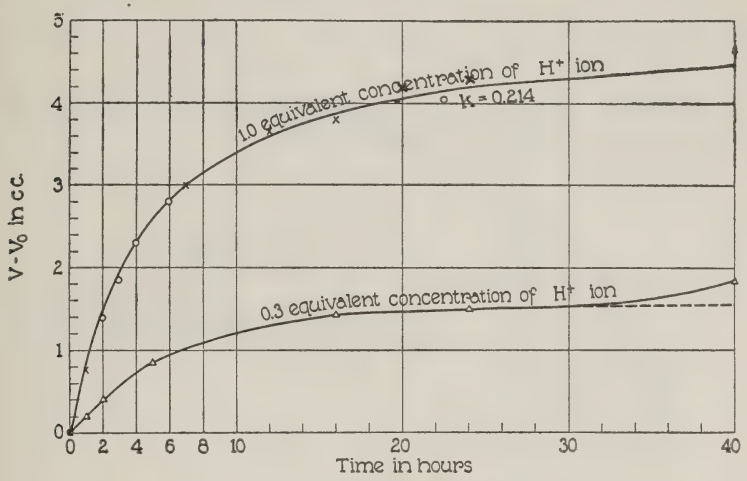

FIG. 1. Hydrolysis of glycyl-glycine.

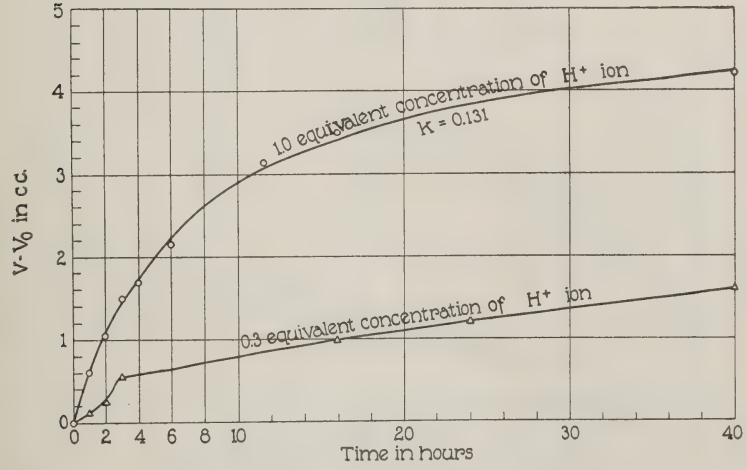

FIG. 2. Hydrolysis of sarcosyl-glycine.

The end-point in the titration of the experiments with glycyl-glycine was the color obtained by titration of a sample of glycine

and the end-point used in the titration of the sarcosine dipeptides was the color obtained by the complete titration of a sample of

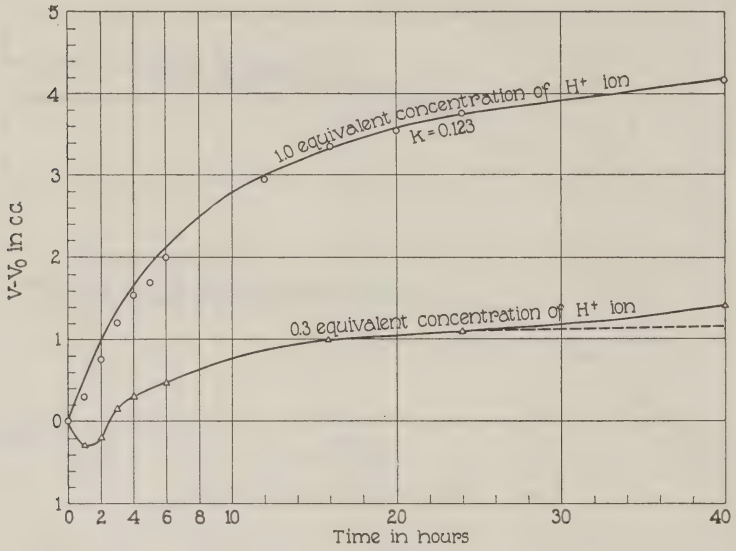

FIG. 3. Hydrolysis of glycyl-sarcosine.

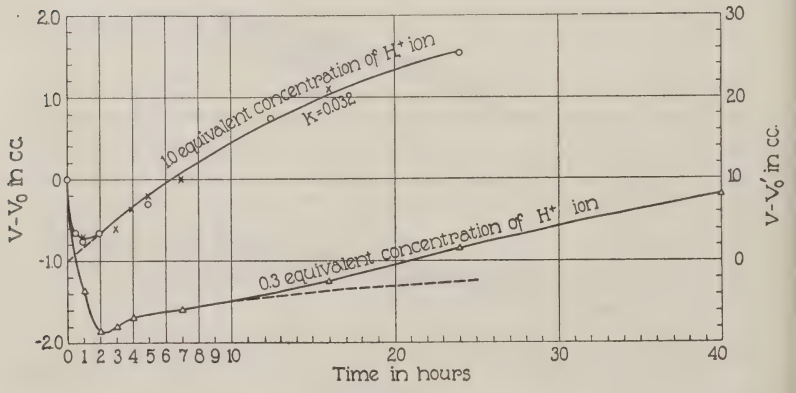

FIG. 4. Hydrolysis of sarcosyl-sarcosine.

sarcosine. Near the end-point the volume was always made up to about 25 cc., as the color of the indicator varies considerably with its concentration. It was necessary in this titration to match the

colors very accurately. At the end-point the solution is orange-red with a slight yellow ring about the meniscus, visible from above (against a white background).

Determination of Dissociation Constants.

The dissociation constants of glycine, sarcosine, and the four dipeptides formed from them, were determined by constant

TABLE XII.

Titration of 0.1 Molar Glycine at 25.0°C.

0.10 M HCl = pH 1.040. Potential of saturated KCl junction assumed constant.

(1)	(2)	(3)	(4)	(5)
$\frac{B}{C}$	pH	B′	pK_1	pK_2
−0.500	2.40	−0.460	2.33	
−0.400	2.55	−0.372	2.32	
−0.300	2.73	−0.281	2.32	
−0.200	2.96	−0.189	2.33	
−0.100	3.39	−0.095	2.33	
0	5.93			
0.100	8.74	Same as Column 1,		9.69
0.200	9.04			9.64
0.400	9.45			9.63
0.500	9.59			9.59
Average			2.33	9.64
$K_a \times 10^3 = 10^{3-pK_1}$		=	4.68	
$K_b \times 10^5 = 10^{5+pK_2-pKw}$		=		7.24

volume titration, using the water-jacketed electrode previously described.[5] The results are presented in Tables XII to XVII and are summarized in Table I. The pK values were calculated by treating pK_1 as if it were the pK of a base and pK_2 as if it were that of an acid, by the formulas:

[5] Simms, H. S., *J. Am. Chem. Soc.*, 1923, xlv, 2503.

TABLE XIII.

Titration of 0.05 Molar Sarcosine at 25.0°C.

0.10 M HCl = pH 1.040. Potential of saturated KCl junction assumed constant.

(1)	(2)	(3)	(4)	(5)
$\frac{B}{C}$	pH	B′	pK_1	pK_2
−0.900	1.98	−0.690	(2.33)	
−0.800	2.08	−0.634	(2.32)	
−0.700	2.18	−0.568	(2.30)	
−0.600	2.27	−0.492	2.25	
−0.500	2.38	−0.416	2.23	
−0.400	2.52	−0.340	2.23	
−0.300	2.68	−0.258	2.22	
−0.200	2.90	−0.176	2.23	
−0.100	3.23	−0.088	2.22	
0.100	9.07	0.100		10.02
0.200	9.40	0.200		10.00
0.300	9.65	0.299		10.02
0.400	9.85	0.398		10.03
0.500	10.01	0.497		10.02
0.600	10.19	0.596		10.02
0.700	10.35	0.694		9.99
0.800	10.57	0.788		10.01
0.900	10.75	0.881		(9.88)
Best average.....			2.23	10.01
$K_a \times 10^3 = 10^{3-pK_1}$ =			5.89	
$K_b \times 10^5 = 10^{5+pK_2-pK_w}$ =				17.0

$$\alpha_1 = -B' = -\frac{H+B}{C}$$ for tritration of pK_1 with HCl,

$$\alpha_2 = B' = \frac{B-OH}{C} = \frac{B}{C} - \frac{K_w}{HC}$$ for titration of pK_2 with NaOH where

B = molar concentration of strong base (NaOH),
−B = " " " " acid (HCl),
C = " " " substance,
H = " " " H ion } neglegible in above equations
OH = " " " OH ion } ln neutral solutions,
±B′ = ± corrected equivalents of strong base (or acid),

and,

TABLE XIV.

Titration of 0.1 Molar Glycyl-Glycine at 30°C.

0.10 M HCl = pH 1.040. Potential of saturated KCl junction assumed constant.

(1)	(2)	(3)	(4)	(5)
$\frac{B}{C}$	pH	B′	pK_1	pK_2
−1.000	2.07	−0.915	3.10	
−0.900	2.34	−0.855	3.11	
−0.800	2.39	−0.774	3.12	
−0.700	2.81	−0.683	3.14	
−0.600	2.96	−0.589	3.12	
−0.500	3.12	−0.483	3.09	
−0.450	3.22	−0.444	3.12	
−0.400	3.31	−0.395	3.12	
−0.300	3.50	−0.297	3.12	
−0.200	3.77	−0.198	3.16	
−0.100	4.10	−0.099	3.14	
−0.050	4.43	−0.050	3.15	
0	5.68			
0.050	6.79	Same as Column 1.		8.07
0.100	7.17			8.05
0.200	7.44			8.04
0.300	7.64			(8.01)
0.400	7.90			8.07
0.450	7.98			8.06
0.500	8.08			8.08
0.600	8.26			8.08
0.700	8.46			8.09
0.800	8.67			8.07
0.900	8.99			8.04
1.000	9.68			
1.100	11.61			
Average pK.....			3.12	8.07
$K_a \times 10^3 = 10^{3-pK_1}$		=	0.759	
$K_b \times 10^5 = 10^{5+pK_1-pK_w}$		=		0.195

TABLE XV.

Titration of 0.05 Molar Sarcosyl-Glycine at 25.0°C.

0.10 M HCl = pH 1.040. Potential of saturated KCl junction assumed constant.

(1)	(2)	(3)	(4)	(5)
$\frac{B}{C}$	pH	B′	pK_1	pK_2
−0.400	3.28	−0.385	3.08	
−0.300	3.48	−0.293	3.10	
−0.200	3.70	−0.196	3.10	
−0.100	4.06	−0.098	3.10	
0	5.10			
0.100	7.58	Same as Column 1.		8.53
0.200	7.89			8.49
0.300	8.14			8.51
0.400	8.34			8.51
Average			3.10	8.51
$K_a \times 10^3 = 10^{3-pK_1}$		=	0.794	
$K_b \times 10^5 = 10^{5+pK_2-pK_w}$		=		0.537

$$pK_1 = pH + \log \frac{\alpha_1}{1-\alpha_1}$$

$$pK_2 = pH + \log \frac{1-\alpha_2}{\alpha_2}$$

Fig. 5 shows the dissociation diagrams of the two amino acids and the four dipeptides. The dotted lines joining the pK values in Fig. 5 *B* show the changes in dissociation produced by substitution of a methyl group on: (1) the free amino group, (2) the bound amino group, and (3) on both groups, of glycyl-glycine.

TABLE XVI.

Titration of 0.05 Molar Glycyl-Sarcosine at 25.0°C.

0.10 M HCl = pH 1.040. Potential of saturated KCl junction assumed constant.

(1)	(2)	(3)	(4)	(5)
$\frac{B}{C}$	pH	B′	pK_1	pK_2
−0.900	2.26	−0.790	2.83	
−0.800	2.40	−0.720	2.81	
−0.700	2.57	−0.646	2.83	
−0.600	2.71	−0.561	2.81	
−0.500	2.88	−0.474	2.84	
−0.400	3.03	−0.381	2.82	
−0.300	3.22	−0.288	2.83	
−0.200	3.45	−0.193	2.83	
−0.100	3.82	−0.097	2.85	
0.100	7.56	Same as Column 1.		8.51
0.200	7.93			8.53
0.300	8.18			8.55
0.400	8.36			8.54
0.500	8.57			8.57
0.600	8.73			8.55
0.700	8.91			8.54
0.800	9.15			8.55
0.900	9.47			8.52
Average			2.83	8.54
$K_a \times 10^3 = 10^{3-pK_1}$		=	1.48	
$K_b \times 10^5 = 10^{5+pK_2-pK_w}$		=		0.576

TABLE XVII.

Titration of 0.05 Molar Sarcosyl-Sarcosine at 25.0°C.

0.10 M HCl = pH 1.040. Potential of saturated KCl junction assumed constant.

(1)	(2)	(3)	(4)	(5)
$\frac{B}{C}$	pH	B′	pK_1	pK_2
−0.800	2.44	−0.728	2.87	
−0.600	2.74	−0.564	2.85	
−0.400	3.06	−0.383	2.85	
−0.200	3.48	−0.194	2.86	
0	6.52			
0.200	8.47	Same as Column 1.		9.07
0.400	8.92			9.09
0.600	9.29			9.12
0.800	9.70			9.10
Average.....			2.86	9.10
$K_a \times 10^3 = 10^{3-pK_1}$		=	1.38	
$K_b \times 10^5 = 10^{5+pK_2-pK_w}$		=		2.09

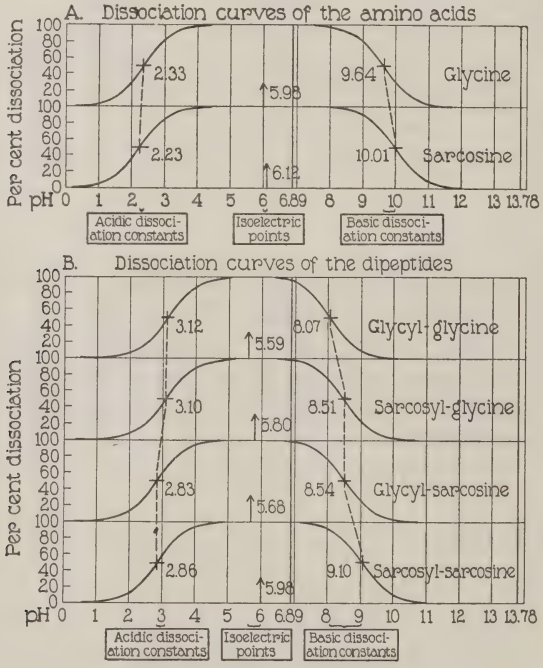

FIG. 5.

ETHER ANESTHESIA.

III. RÔLE OF LACTIC ACID IN THE ACIDOSIS OF ETHER ANESTHESIA.

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That the alkali reserve during ether anesthesia is reduced from 13 to 34 volumes per cent and that this condition is accompanied by an increase in the hydrogen ion concentration of the blood have been shown by a number of investigators, Van Slyke, Austin, and Cullen (1922). It has recently been shown that these changes take place early in anesthesia and that anoxemia alone is not responsible (Cullen, Austin, Kornblum, and Robinson (1923)). That they are not due to the production of acetone bodies has been demonstrated by Leake, Leake, and Koehler (1923), and by Gramén (1922) who found no acetone during prolonged anesthesias. Leake interprets his findings on acetone bodies as evidence against the formation of other acid products. This would leave withdrawal of base from the blood as the alternative explanation. The accumulation of lactic acid in the blood does not seem to be excluded as the possible cause of changes in the acid-base equilibrium. The importance of lactic acid in the acidosis of muscular exercise and the influence oxidative processes have on its accumulation suggest it as possibly responsible for these changes.

In this paper an attempt has been made to correlate changes in the lactic acid content of the blood with changes in the acid-base equilibrium, and at the same time to show whether or not the amount in the blood depends on the pH as controlled by the CO_2 tension, in this condition, as has been found by Anrep and Cannan (1923) in experimental acidemia and alkalemia. An effort was made to throw light on the possible source of lactic

acid and the cause of its accumulation. The degree of cyanosis or the oxygen content of capillary blood is the nearest index we have of the oxygen supply to the tissues. A comparison of oxygen content of arterial and venous blood has been studied in an attempt to determine the condition of tissues in regard to the oxygen supply.

Procedure.

Animals.—Dogs, weighing between 10 and 15 kilos, were used, a size that could be easily handled by two persons and sufficiently large that the withdrawal of 150 to 200 cc. of blood would not seriously disturb the condition of the animal. The animals were fed as usual on the the day preceding, but received no food on the day of the experiment. They were placed in a cage the evening before and kept as quiet as possible until the experiment was started. The dogs were tied to the board, usually without struggling, and the initial sample of blood was taken. Ether was then administered. In all cases the normal temperature of the dog was maintained by means of a heating pad controlled by a thermostat placed in the rectum of the dog, as described by Bishop (1923).

Induction of Anesthesia.—Anesthesia was induced by the drop method. A wire cone covered with gauze was placed over the nose of the animal. Even with the greatest care we found ourselves unable to produce anesthesia without some excitement and struggling on the part of the animals, Experiments I, II, and III. To reduce this to a minimum we found the best method was to decrease the period of induction. Since the concentration of ether in the blood depends on the alveolar tension, and since the stimulus to respiration occasioned by increasing the CO_2 tension had already been shown to increase the rate of elimination of ether (Haggard and Henderson (1919), White (1923), Ronzoni (1923)), it naturally occurred to us that induction could be hastened in the same way. This has recently been demonstrated by Haggard (1924) to be true. The CO_2 concentration was increased by using a close fitting mask and allowing the animal to rebreathe into the mask until the rate of respiration was increased before pouring on the ether. If the respiration was first stimulated there was no reflex inhibition due to irritation of the mucous membrane of the nasal passage by ether. This procedure also decreased the oxygen tension, but since the whole procedure was over in a period of about 2 minutes this was thought to have little effect on the subsequent blood reactions. Analysis of the air in the mask showed from 6 to 8 vols. per cent CO_2 and from 15 to 18 vols. per cent O_2. The struggling was vigorous, but of short duration.

After induction, tracheotomy was performed and the animals were attached to the ether apparatus described in a previous paper (Ronzoni (1923)). If the animal was to recover the mask was replaced by a few layers of gauze to insure adequate ventilation and the drop method continued. In Experiments VI and VII the mask was used. The oxygen unsaturation

of the blood in the early part of these experiments shows that the ventilation was interfered with. In Experiment VIII no mask was used. The increased O_2 unsaturation was due to depression of respiration accompanying the deep anesthesia.

Collection of Samples.—Blood was drawn from the femoral artery into a syringe previously coated with and containing about ½ inch of oil. The initial sample in each case was taken through the skin and in those cases where the animal was allowed to recover subsequent samples were drawn in the same manner. Otherwise the artery was exposed and the blood taken by needle and syringe. When removing the needle the circulation was stopped for a few seconds by gentle pressure, thus allowing time for the closing of the puncture hole. The blood was then allowed to resume its flow. This procedure necessitated no part of the body being cut off from the general circulation for more than a few moments.

The blood was transferred from the syringe under oil to a tube containing enough neutral oxalate to make 0.3 per cent and sodium flouride to give a concentration of 0.05 per cent, shown by Evans (1922) to prevent production of lactic acid. After collection the blood was placed in the refrigerator until analyzed. That there was no significant change in the blood during the interval between drawing and analysis is shown by the fact that there was no measurable change in pH. Blood to be used for ether determinations was drawn directly into a pipette graduated in cubic centimeters. After enough blood for duplicate determinations was collected this was measured directly into aeration flasks containing oxalate. The last portion of blood, the layer exposed to the air, was discarded. The aeration tubes of the flasks were closed until aeration was started—within a few minutes after drawing the blood.

Blood gas analyses were made on whole blood in the Van Slyke constant pressure apparatus, using the technique described by Van Slyke and Stadie (1921). The O_2 and CO_2 contents of the blood as drawn were determined. A 5 cc. sample was equilibrated with an air and CO_2 mixture, containing approximately 5 vols. of CO_2 and 19.5 vols. per cent O_2. These equilibrations were carried out in liter bottles at a temperature between 36° and 38°C. The blood was removed under oil without exposure to air. The gas mixture was trapped in the bottle and drawn out into a Haldane gas apparatus under reduced pressure for analysis. Thus the actual tension of CO_2 and O_2 with which the blood was in final equilibrium was determined. Analysis of a sample of blood so equilibrated gives both the CO_2 and O_2 capacity of the blood, proper correction being applied for the amount of physically dissolved O_2. All gas analyses were made in duplicate, usually by two observers.

The possible effect of ether contained in the blood on the blood gas determinations suggested itself to us early in the investigation. We found that concentrations of ether similar to those existing in the blood of anesthetized animals caused a considerable error in the CO_2 determinations as has also been reported by Austin (1924). An investigation of the conditions of our experiments shows this error to be actually negligible. As

already described the blood was collected in an oil-coated syringe and allowed to stand for some time under oil before analysis. The tubes used for collection were 2 cm. in diameter, and, since whole blood was used, stirring was necessary to mix the corpuscles and plasma before each sample was measured. Examination of the blood after the samples had been removed for gas analyses showed a great reduction in the amount of ether in the blood. This is to be expected from the fact that the partition coefficient for ether between oil and blood is greatly in favor of the oil. Table I gives data from a series of bloods examined; the maximum ether found in blood at the time the gas analyses were made was 45 mg. per 100 cc. This amount of ether added to a bicarbonate solution of a known strength increased the apparent volume of CO_2, obtained from 1 cc. of the solution, by about 0.01 cc. If the total volume of CO_2 contained in 1 cc. of blood amounted to 0.5 cc., then the error due to CO_2 would be 2 per cent and the apparent CO_2 content would be 2 per cent too high. The lower the CO_2 content the greater the error due to ether. Since the CO_2 content of blood

TABLE I.

Amount of Ether Contained in 100 Cc. of Blood, Calculated from the Distribution Ration and Relative Volumes of Blood and Air.

Ether per 100 cc. of blood.		
When drawn.	After samples have been removed for gas analyses.	After equilibration.
mg.	*mg.*	*mg.*
162	45.0	11.3
183	41.3	12.7
196	38.5	13.7
132	40.2	9.4

as drawn falls as low as 25 vols. per cent the error might be increased to 4 per cent.

In the equilibration for the determination of CO_2 capacity the volume of gas with which the blood was in equilibrium was 200 times that of the blood used. The distribution ratio for ether between air and blood at 38° is 1:15. So the blood after equilibration would contain only 1/14 the total ether, or in the case of blood originally containing 162 mg. per 100 cc., 11.3 mg., an amount too small to have a measurable effect.

Values for CO_2 content of blood as drawn may therefore be from 2 to 4 per cent too high; and the apparent changes less than those really existing. These errors would be reflected in the calculated values of CO_2 tension. Such errors fall easily within the limits of error in lactic acid determinations. The values for CO_2 capacity of the blood are not influenced by ether since the amounts present caused no measurable effect in CO_2 determination.

The pH of the blood was determined by Cullen's (1922) colorimetric method. Clausen's (1922) method was used for lactic acid. This deter-

mination often had to be left till the following day. In all cases the blood filtrates, however, were made immediately after drawing the blood, the sugar was precipitated, the filtrate placed in the refrigerator, and analyses were made within the next 24 hours, a procedure which has been found to be safe. To eliminate the possibility of the ether in the blood affecting the determination of lactic acid or the possible influence of acetone bodies, the filtrates after having been measured for lactic acid determinations were acidified and aerated for 15 minutes at a temperature of 100°. Samples that were aerated showed the same lactic acid content as similar samples unaerated, which shows that the ether has no effect and that acetone does not accumulate. In fact, the lactic acid of the aerated sample always ran a trifle higher than that of the unaerated. The differences were well within the limits of error of the lactic acid method, but were always in the same direction, an observation which we are unable to explain.

Ether determinations were made by a modification of the Nicloux method described by Shaffer and Ronzoni (1923). Blood sugar determinations were made on all samples, but these results will be discussed in a future paper.

Calculation of Data.

In all cases the pH and CO_2 content of the blood were determined. The CO_2 tension was calculated by means of Hasselbalch's equation

$$pH = pK_1 + \log \frac{BHCO_3}{H_2CO_3}$$

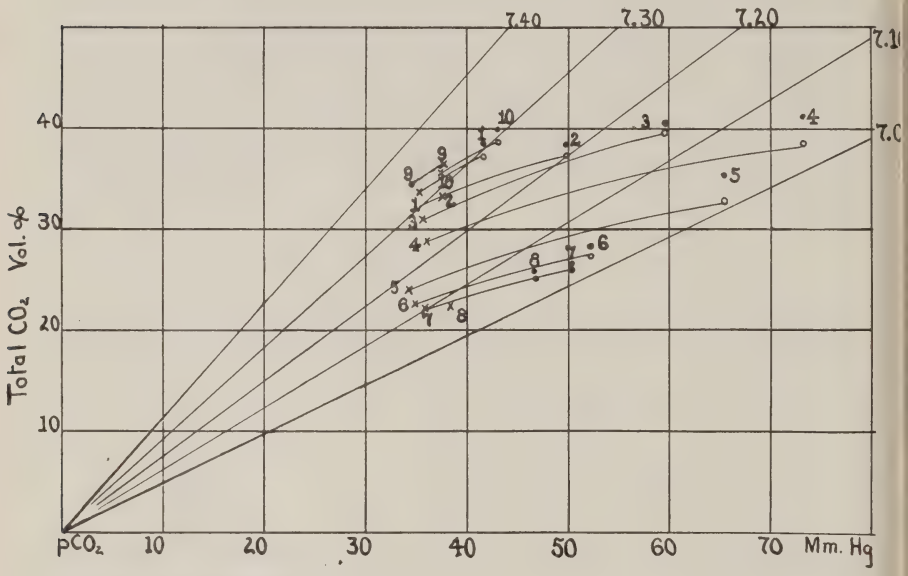
$$H_2CO_3 = \frac{\alpha p\, 100}{760} = 0.01316\, \alpha p$$

$$BHCO_3 = CO_2 - 0.1316\, \alpha p$$

$$CO_2 \text{ tension} = \frac{CO_2}{0.1316\ \alpha\ (10^{pH-pK_1} + 1)} \left(\begin{array}{c} \text{Austin, Cullen, Hastings, Mc-} \\ \text{Lean, Peters, and Van Slyke} \\ (1922) \end{array} \right)$$

p is partial pressure of CO_2. For α, the solubility coefficient of CO_2 in whole blood, Bohr's (1905) value 0.511 was used. 6.20 was taken as the value of pK_1, this having been determined on dog's blood by Van Slyke and coworkers. Samples of the same bloods were also equilibrated with a known tension of CO_2 and the CO_2 capacity was determined. This gives the data for the general slope of the absorption curve of fully oxygenated blood when correction is made for O_2 unsaturation of the blood as drawn. These were plotted on CO_2 diagrams introduced by Haggard and Henderson (1919) and the volume per cent CO_2 read off at a

constant CO_2 tension 38 mm. for purposes of comparing the changes occurring in the alkali reserve. Corrections for the oxygen unsaturation of the arterial blood as drawn were made by the formula of Peters, Barr, and Rule (1921), $K \times \text{Hb} = D$, where K is a constant, Hb is oxygen unsaturation expressed in volumes per cent, and D is the change in level of absorption curve expressed in volumes per cent of CO_2. The value for K of 0.27 was used as



● CO_2 content as drawn.
○ Corrected for O_2 unsaturation.
× Equilibrated sample.

Chart I. Data from Experiment VII.

determined by Doisy, Briggs, Eaton, and Chambers (1922). Chart I, from the data of Experiment VII, shows the graphic method.

The changes in pH, CO_2 tension, alkali reserve at a constant CO_2 tension, and lactic acid, expressed in terms of lowering of CO_2 capacity, are plotted for all experiments. Protocols are given in the tables and show the determined and calculated values on which the diagrams are based.

RESULTS AND DISCUSSION.

Relation of Lactic Acid to Changes in Alkali Reserve.—The accumulation of lactic acid in the blood during anesthesia accompanies the fall in pH and in the alkali reserve as measured by the CO_2 capacity at a constant CO_2 tension. The relation between lactic acid increase and the fall in CO_2 capacity of the blood may be seen in Charts II, III, and IV, and is summarized in Table II. Assuming that 4 mg. of lactic acid reduce the CO_2 capacity 1 volume per cent, the accumulation of lactic acid does not correspond to the fall in alkali reserve. That this is an important factor, however, is obvious. It is not to be expected that even though the accumula-

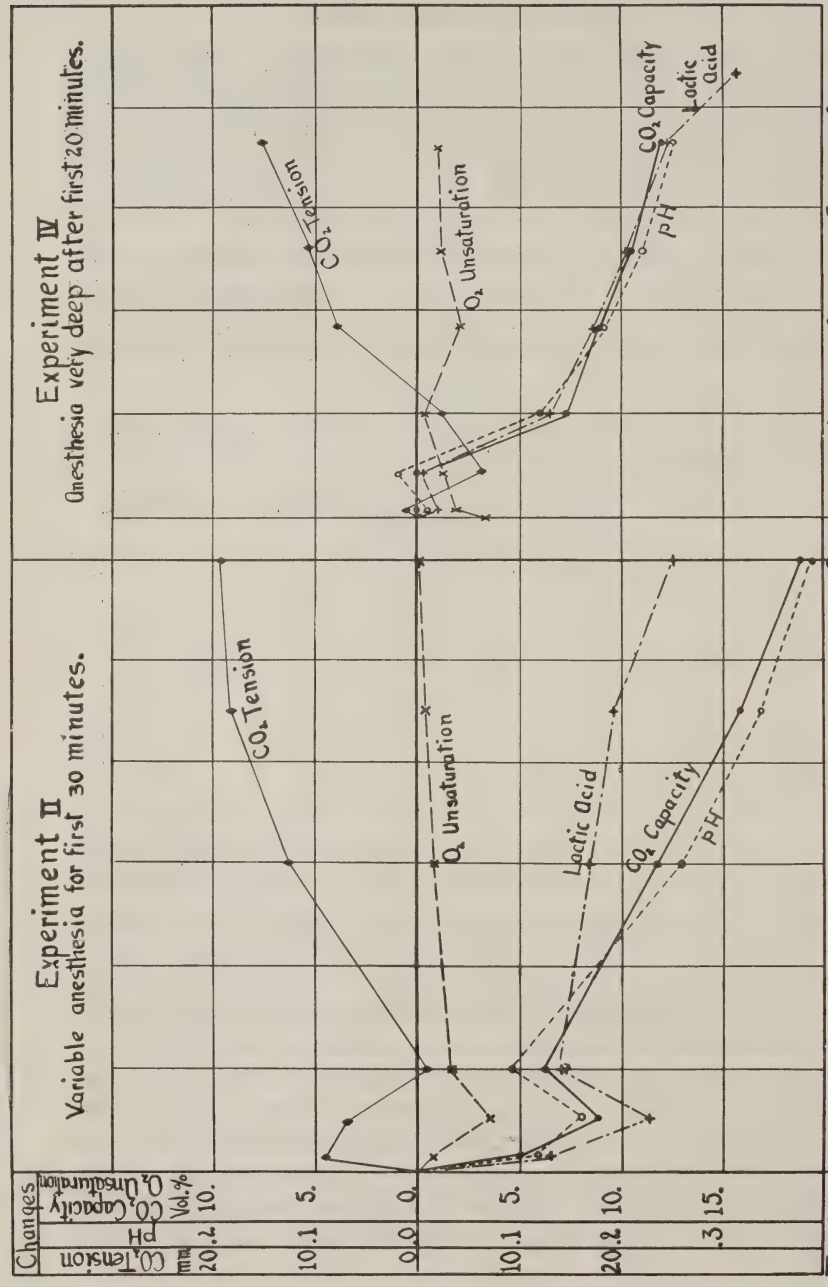
TABLE II.

Fall in CO_2 Capacity Determined and Calculated from Lactic Acid.

Experiment No.	Lactic acid increase.	Fall in CO_2 capacity.			Decrease in pH.	Duration of anesthesia.	
		Determined.	Calculated.	Difference.			
	mg.	*vol. per cent*	*vol. per cent*	*per cent*		*hrs.*	*min.*
II	50.6	18.7	12.6	−33.2	0.49	5	0
III	33.7	15.3	8.4	−44.1	0.29	1	45
IV	49.2	12.2	12.3	+1.0	0.25	3	40
V	37.0	9.0	9.2	+2.2	0.24	2	5
VI	41.9	15.4	10.5	−31.8	0.25	2	5
VII	64.0	13.0	16.0	+23.1	0.27	2	0
VIII	30.5	12.0	7.6	−36.7	0.37	1	0
IX	41.9	10.5	10.5	±0.0	0.18	2	0

tion of lactic acid was entirely responsible for the change in CO_2 capacity and pH, the amount in the blood at any one time would exactly correspond, since undoubtedly the lactic acid is produced in the tissues and must be in higher concentration there than in the blood until equilibrium had been reached. At any time there might be a withdrawal of base from the blood to the tissues without a corresponding increase of lactic acid apparent in the blood.

Recent experiments of Stehle and Bourne (1924) show that phosphoric acid leaves the muscles during anesthesia and sojourns in the liver until the reassumption of kidney function after the recovery of the animal; and since the transport of this must be through the blood, it is suggested that the increased acidity of the blood is due to the discharge of phosphoric acid from the muscle.


Experiment II
Variable anesthesia for first 30 minutes.
Experiment IV
Anesthesia very deep after first 20 minutes.
Changes
CO2 Tension
pH
CO2 Capacity
O2 Unsaturation
mm
Vol.%
CO2 Tension
O2 Unsaturation
Lactic Acid
CO2 Capacity
pH
Time, hours
Ether on
Ether on

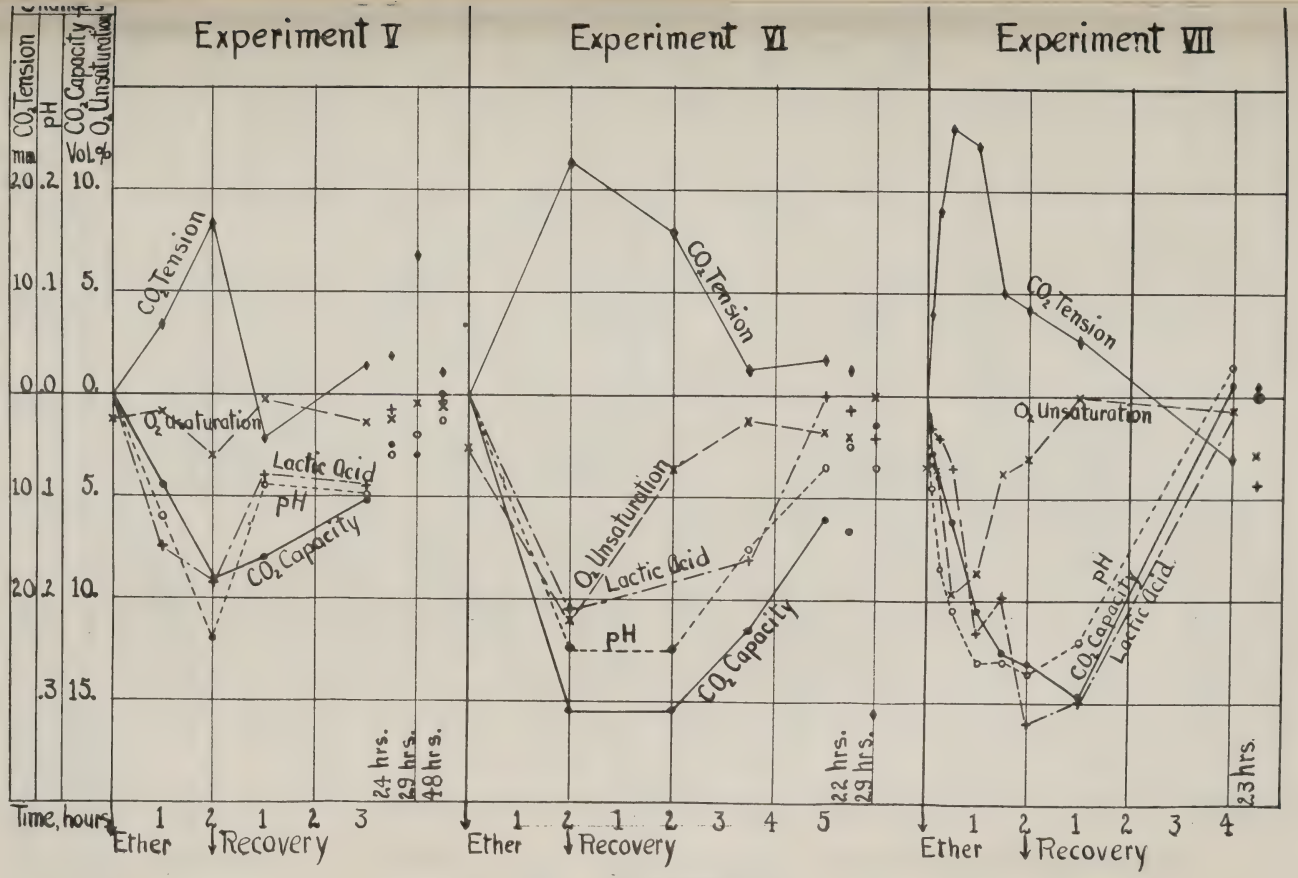
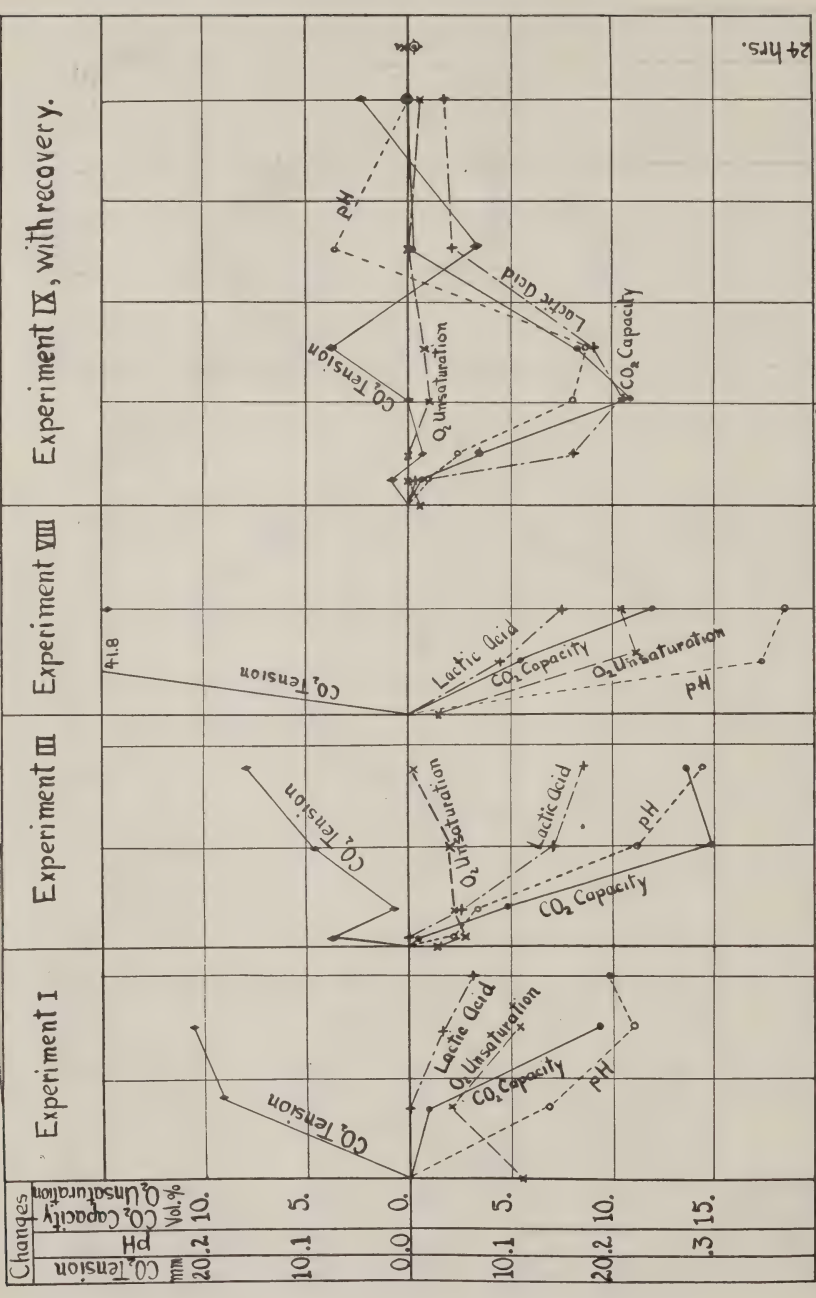

Experiment V
Experiment VI
Experiment VII
CO2 Tension
pH
CO2 Capacity
O2 Unsaturation
mm.
Vol. %
Lactic Acid
24 hrs.
29 hrs.
48 hrs.
22 hrs.
23 hrs.
Time, hours
Ether
Recovery

CHART III.


Experiment I
Experiment III
Experiment VIII
Experiment IX, with recovery.
Changes
CO2 Tension
pH
CO2 Capacity
O2 Unsaturation
mm
Vol.%
20.2
10.1
0.0
10.
5.
0.
5.
10.
15.
.3
Time, hours
Ether on
Ether off
24 hrs.
CO2 Tension
Lactic Acid
O2 Unsaturation
CO2 Capacity
pH
4.1.8

Since the changes in blood phosphates were not followed the extent to which this would actually account for changes in the alkali reserve of the blood is not known. The same investigators state that they found no excretion of lactic acid in the urine during or after anesthesia. This is not a good criterion of conditions existing in the blood since it is a well known fact that only a small part of the lactic acid present in the blood is excreted by the kidney, and besides, the kidney function is markedly interfered with by anesthesia.

In Experiments II and IV, Chart II, Experiment III, Chart IV, and Experiment VII, Chart III, where the early stages of anesthesia were followed closely, we did not find such constant and abrupt changes as reported by Cullen, Austin, Kornblum, and Robinson (1923). With the exception of Experiment IV each case shows a fall in CO_2 capacity as early as 4 minutes after starting the ether. In Experiment II there was marked struggling of the animal while ether was being administered and during the first half hour the anesthesia was so light that all reflexes were present and the animal trembled considerably. This would account for the fact that the alkali reserve fell abruptly, accompanied by an accumulation of lactic acid, then rose again when a steady state of anesthesia was reached. At least part of the initial fall in pH can be accounted for by the increasing CO_2 tension in the early part of the experiment which was caused by choking and reflex inhibition of respiration. A fall in CO_2 tension accompanied the establishing of regular respiration when narcosis was complete. During the last $1\frac{1}{2}$ hours of the experiment the concentration of ether was increased. Respiration was depressed and the CO_2 tension showed a continued rise, but the O_2 unsaturation was not increased. There was a continued drop in the alkali reserve, but no indication of an increase in the rate of fall due to increasing the depth of anesthesia. The lactic acid showed a slightly more rapid accumulation with high concentration of ether. Experiment IV shows a similar effect due to early irregularities in anesthesia. The first drop in pH seems to be due to a rise in CO_2 tension caused by reflex inhibition of respiration. The subsequent rise in pH without change in CO_2 capacity is due largely to the marked fall in CO_2 tension. The early changes in lactic acid are slight enough to be within the limits of accuracy of the method and are not significant. By

the time the third sample was drawn the anesthesia had been regulated so that from this point the concentration in the blood remained constant and high. The respiration was not depressed, but remained almost constant until it stopped rather abruptly in 4 hours and 20 minutes. The heart continued beating for about 4 minutes after respiration failed. The last sample of blood was drawn from the heart.

During recovery there is a complete return to normal in from 3 to 5 hours. The lactic acid, CO_2 capacity, and pH all return at the same rate. Experiments V, VI, and VII, Chart III, were all done on the same animal at intervals of 1 week. In each experiment the depth of anesthesia was the same as shown by the ether concentration. In Experiment V the fall in CO_2 capacity was less marked than in either of the subsequent experiments, as is the accumulation of lactic acid. The fact that the O_2 capacity and the level of the initial absorption curve remained the same in the three experiments indicates that the general condition of the animal had not been interfered with by repeated bleedings and anesthesias. The possible relation to O_2 tension and of CO_2 tension and pH to lactic acid accumulation will be discussed later.

In Experiment IX, at the end of the 1st hour of recovery, though the lactic acid was less, the CO_2 capacity had increased, and the pH was lower than at the end of the anesthesia. This fall is accounted for by the rise in CO_2 tension. In this dog there was a marked secretion of saliva. The dog choked several times during the experiment and undoubtedly swallowed a large amount of saliva, containing ether. This was the only case of severe vomiting after anesthesia and suggests the presence of ether in the stomach as the cause of vomiting.

On the day following the experiment there was no indication of acidosis in any of the four cases observed. Tests for acetone bodies in the urine were negative. This would point to the fact that the acidosis clinically observed after operations is postoperative rather than postanesthetic and is not caused by the ether, but is rather referable to the condition of the patient or to the shock caused by the operation.

Concentration of Ether in Relation to Production of Lactic Acid.— Variations in concentration of ether between the limits that will

abolish reflexes and those that will produce respiratory failure seem to have little influence on the rate of production of lactic acid, other factors remaining constant. In very light anesthesia when consciousness alone is lost and reflexes are still present, movements on the part of the animal affect the lactic acid content of the blood. In Experiment II there was trouble in adjusting the ether concentration so that for the first half hour anesthesia was very light. The animal trembled violently for a few minutes after the initial violent struggling. The lactic acid in this case was increased during the first 30 minutes, then fell with a subsequent rise. When anesthesia is so deep that respiration is depressed as in Experiments IV and VIII the accumulation of lactic acid is not greater than in other experiments. The high concentration of ether in the blood in Experiment III was apparently due to a progressive lipemia, since the concentration of ether remained constant in the air breathed. The increased concentration of ether in the blood was due to a change in the distribution ratio. This has no effect on the lactic acid production. Table III summarizes the data obtained from various experiments.

CO_2 Tension.—With the exception of Experiments IV, IX, and during the second 30 minutes of Experiment II, the CO_2 tension during the administration of ether is greater than when the initial sample was drawn. That the lactic acid accumulation during anesthesia is not due to an increase of pH caused by a fall in CO_2 tension is obvious from these experiments, Table IV. In only one case is there an increase in pH during any part of the experiment. Cullen and coworkers (1923) found no increase in pH at any time during the early stages of anesthesia when following the blood reaction very closely. Under the conditions of ether anesthesia, then, the contentions of Anrep and Cannan (1923) that the accumulation of lactic acid in the blood is controlled by the pH seem not to be justified.

Reduced Oxidation.—The cause of the accumulation of lactic acid during ether anesthesia becomes a subject of interesting speculation. The amount of lactic acid in the blood is determined not only by its rate of production but by the rate of removal. Muscular contraction involves the production of lactic acid and if its removal cannot keep pace with the increased rate of production, lactic acid accumulates in the blood and other tissues of the

body. Assuming muscular activity to be the source of lactic acid, the fact that it is reduced to a minimum during anesthesia suggests that increased production does not explain its accumulation. The fact that narcotics follow the Meyer-Overton law as de-

TABLE III.

Relation of Depth of Anesthesia to Accumulation of Lactic Acid.

Experiment No.	Ether in blood.	Increase in lactic acid.		Remarks.
		1 hr.	2 hrs.	
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	
II*	145	27.0	36.0†	Anesthesia variable.
III	130–213	27.7	33.7	Increasing concentration of ether in blood. Tension constant.
IV	160	25.7	36.4†	Deep anesthesia.
V	128	32.0†	37.0	Moderate anesthesia.
VI	126	28.0†	41.9	" " low O_2 tension.
VII	120	46.5	64.0	" " " " "
VIII	183	30.5		Deep anesthesia.
IX	115	31.8	41.9	Moderate anesthesia.

*Experiment I omitted since no initial sample of blood was obtained.
†Determination not made on exact hour. These values were interpolated.

TABLE IV.

Changes in CO_2 Tension, pH, and Lactic Acid at End of 1 Hour of Anesthesia.

Experiment No.	CO_2 tension change.	pH decrease.	Lactic acid increase.
	vol. per cent		*mg. per 100 cc.*
II	−1.0	0.09	27.0
III	+8.7	0.23	27.7
IV	−2.7	0.12	25.7
V	+9.5*	0.16*	32.0*
VI	+12.0*	0.12*	20.9*
VII	+23.9	0.26	46.5
VIII	+29.4	0.37	30.5
IX	−1.3	0.05	31.8

*These values were interpolated.

pressants of oxidation, as they do in their activity as anesthetics, that is, the higher members of a homologous series work in smaller concentrations (Warburg and Wiesel (1912)), leads to the suggestion that we have here an interference with oxidation, and therefore,

a decrease in the rate of removal which would account for the accumulation of lactic acid in the blood.

In Experiment II the removal of lactic acid formed in the first half hour accompanying, presumably, increased muscular activity, would indicate that the ability of the body to remove lactic acid had not been completely impaired by the anesthesia.

Oxygen Unsaturation of Arterial Blood.—This was determined in an attempt to throw light on the conditions of the tissues in regard to oxygen supply. In Experiments V, VI, and VII the same animal was used. Experiment V shows the least degree of oxygen unsaturation and the smallest increase of lactic acid in the blood. However, we find on examining Experiments VI and VII that in No. VI, where the oxygen unsaturation was as much as 11.7 volumes per cent or 59 per cent of the total oxygen capacity, that the lactic acid increase was 41.9 mg. whereas in No. VII, where the oxygen unsaturation varied between 3 and 9 volumes per cent or between 15.8 and 47 per cent of the total oxygen capacity, the lactic acid increase was much greater, 64.0 mg. per 100 cc. of blood. In these experiments the oxygen unsaturation was due to mechanical interference with ventilation. In Experiment VIII the respiration was depressed with a marked unsaturation of the arterial blood. At the end of 1 hour the lactic acid had increased 30.5 mg. over the initial value. However, in Experiment IX, where the hemoglobin of the blood was 100 per cent saturated during the 1st hour 31.8 mg. of lactic acid accumulated per 100 cc. of blood. Oxygen unsaturation of arterial blood, then, does not appear to be the influencing factor.

The oxygen content of arterial blood is no adequate index of the oxygen environment of the tissues. The rate of circulation and the degree of unsaturation of capillary blood would be a better index. To investigate this, a determination was made of the relative amounts of oxygen removed from the blood in passing through the tissues before and during anesthesia, by determining the oxygen content of arterial and venous blood drawn at the same time. The amounts removed varied considerably both before and during anesthesia as shown in Table V, but show no striking differences. If the blood flow through the tissues were greatly reduced, one would expect more oxygen to be removed in passage through the tissues unless ether prevented the utilization of oxygen.

The possibility of ether influencing the diffusion of oxygen seems to be ruled out by the fact that the diffusion from the lungs to the blood is not interfered with. It would seem probable from these figures that the oxygen supply to the tissues is not interfered with and that the accumulation of lactic acid during ether narcosis is an

TABLE V.

Comparison of O_2 Content of Venous and Arterial Bloods Drawn as Nearly at the Same Time as Possible.

The venous sample was always drawn first, the arterial immediately following. Blood pressure recorded from carotid artery.

Time.	Source of blood.	Blood pressure.	Pulse rate.	O_2 chemically bound.			Lactic acid of arterial blood.
				Arterial.	Venous.	Difference.	
a.m.		*mm. Hg*	*per min.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*mg. per 100 cc.*
8.40	Femoral artery and vein.		140	20.2	14.7	5.5	16.8
8.55	" " " external jugular.		140	20.3	16.3	4.0	
9.10	Femoral artery and vein.		140	20.1	12.2	7.9	18.3
9.30	Anesthesia started. Tracheotomy.						
9.40	Attached to ether apparatus 120 mg. per 100 cc. blood.						
11.00	Carotid and internal jugular.	120	140	19.4	16.3	3.1	39.3
11.10	Femoral artery and vein.	120	140	18.2	13.6	4.6	
11.27	Carotid and external jugular.	120	140	19.0	15.1	3.9	
11.35	Femoral artery and vein.	150	160	18.6	12.3	6.3	45.6
11.40	Ether concentration increased to 160 mg. per 100 cc. blood.						
p.m.							
12.20	Femoral artery and vein.	120	160	16.2	12.1	4.1	
12.30	Carotid artery and jugular vein.	110	180	16.4	13.1	3.3	63.8
12.40	Right and left heart.	100	150	15.8	9.4	6.4	

effect of the ether on the tissues themselves or possibly on the central nervous mechanism as is commonly supposed to be the case in the production of hyperglycemia of anesthesia.

In a preliminary attempt to locate the source of lactic acid, the content of blood passing through the leg and through the

liver was determined. The blood from the femoral vein in each of four samples analyzed contained from 10 to 16 mg. more lactic acid than arterial blood taken at the same time. Blood from the hepatic vein showed no such increase over the arterial blood drawn simultaneously. The hepatic venous blood did show an increase in glucose content which suggests a disturbance in hepatic function. The results obtained from hepatic blood cannot be relied on since, even exercising the greatest care, drawing blood from the hepatic vein involves some manipulation of the gastro-intestinal tract and pressure on the liver. This can be reduced to a minimum by raising the animal to a vertical position and allowing the abdominal viscera to drop of their own weight. If the ligaments holding the liver in place are clipped, it falls down leaving the hepatic vein exposed. The fact that the glucose content of the blood went up markedly when this part of the experiment was done would make it impossible to draw conclusions. This, however, did not influence the results on blood from the leg since these were obtained before operative procedures were commenced. In so far as these results can be relied upon the source of the lactic acid is the muscular tissues.

If Embden's (1922) idea that "lactacidogen," a hexose phosphoric acid, is the precursor of lactic acid, is correct, the findings of Stehle and Bourne (1924) showing a loss of phosphoric acid from the muscle and our findings showing a production of lactic acid by the muscle, might be interpreted as a breaking down of "lactacidogen" with a consequent throwing into the blood stream of both lactic and phosphoric acids. The phosphoric acid is taken out by the liver, but the lactic acid remains in the circulation, thus accounting for the greater part of the lowering of the alkali reserve. These observations call to mind those of Embden, Griesbach, and Schmitz (1914) showing that nearly equimolecular amounts of phosphate and lactic acid are produced in muscle juice. Too close an analogy cannot be drawn between these two observations until more quantitative work has been completed.

Since muscle tonus is reduced under ether, the normal production of lactic acid should also be reduced. From the fact that the amount given up to the blood increases, we must conclude, as already pointed out, either that there is an abnormal production not due to tonus nor to muscle activity, but due to the effect of ether,

or else that, due to ether the oxidation of what little is produced is very markedly inhibited and is, therefore, not rebuilt into glucose or other precursor. If, in the normal restoration of lactic acid in the muscle, the phosphoric acid is retained, as oxidative removal of the lactic acid takes place, the fact that it is not retained during anesthesia might indicate that the muscle is producing unusual amounts of lactic acid. If, however, the phosphoric acid is normally excreted, as it is in anesthesia, then the presumption is that the normal production is taking place, but with subnormal oxidative restoration. The increased excretion of phosphates after exercise (Embden, Grafe, and Schmitz (1921)) suggests the latter explanation. If the fall in heat production is responsible for decrease in temperature commonly noted in animals under anesthesia, accompanied as it is by an *increase of lactic acid*, this points to subnormal removal of lactic acid.

CONCLUSIONS.

1. Accumulation of lactic acid accounts in a large part for the acidosis of ether anesthesia.

2. Its increase is independent of CO_2 tension and produces the changes in pH rather than being itself controlled by pH as stated by Anrep and Cannan.

3. Decreased oxygen supply to tissues does not account for its production.

4. The source of lactic acid seems to be the muscle tissues.

5. Production of lactic acid in the muscle, together with loss of phosphate from the muscle (Stehle and Bourne), during anesthesia, points to a breakdown of some hexose phosphate, such as Embden's "lactacidogen."

Protocols of Experiments.

Experiment I.

Moderate Anesthesia.

Female dog. Weight not taken. Very fat. Impossible to get initial sample due to small arteries and struggling. Ether started 10.00 a.m. First sample taken 2 minutes after cone applied. Violent struggling for about 2 minutes. Tracheotomy. Attached to ether apparatus. Sample 4 lost for blood gas determinations. Bleeding from the carotid artery. Total loss of blood 65 cc.

Time.	No.	Ether.	Lactic acid.	pH	Total CO_2 as drawn.		CO_2 of equilibrated sample.			Oxygen chemically bound.	
					Content.	Tension calculated.	Content.	Tension.	Content at 38 mm. CO_2.	Content.	Capacity.
a.m.		*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*
10.02	1	122	51.8	7.32	38.1	39.8	35.5	38	36.0	15.8	21.2
10.40	2	126	50.9	7.18	41.5	58.3	35.8	40	34.5	17.7	19.8
11.30	3	120	58.9	7.10	36.6	59.9	26.6	38	26.6	14.4	19.9
p.m.											
12.00	4	128	66.0	7.12							

Experiment II.

Effect of Variable Anesthesia.

Male dog. Weight 13.2 kilos. Initial bleeding 9.20 a.m., very little struggling. Ether started 9.30 a.m. Animal under in 2 minutes. Tracheotomy. Ether apparatus attached to animal within 10 minutes. Apparatus not adjusted correctly. Concentration of ether was increased during the last half hour. The respiration was depressed, but not enough to cause any difference in the saturation of the blood. Total loss of blood 120 cc.

Time.	No.	Ether.	Lactic acid.	pH	Total CO_2 as drawn.		CO_2 of equilibrated sample.			Oxygen chemically bound.	
					Content.	Tension.	Content.	Tension.	Content at 38 mm.	Content.	Capacity.
a.m.		*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*
9.20	1		21.5	7.36	45.1	43.6	37.5	33.5	41.0	18.0	16.5(?)
9.40	2	108	46.7	7.24	42.8	53.1	35.6	37.5	36.0	19.5	19.9
10.00	3	132	68.4	7.19	36.9	51.0	33.7	41.0	31.8	15.8	19.3
10.30	4	145	48.5	7.27	36.5	42.6	37.3	44.0	34.8	17.8	18.3
p.m.											
12.30	5	135	54.2	7.10	33.9	56.4	28.8	37.2	28.9	18.2	18.8
2.00	6	160	59.6	7.02	31.9	62.3	25.3	37.5	25.1	18.4	18.8
3.45	7	164	72.1	6.97	29.3	62.8	22.2	37.0	22.5	18.2	18.3

Experiment III.

High Ether Concentration Due to Lipemia. Ether Tension Remained Constant.

Female dog. Weight 13 kilos. Dog playful to begin with, no struggling before taking the initial sample. Ether started 9.00 a.m., drop method. Severe struggling for about 1 minute. Tracheotomy. Attached to ether apparatus 9.15 a.m. Blood showed a progressive lipemia accounting for the increased concentration at same tension. Final distribution ratio 1:18.5. Samples 4 and 5 from carotid artery. 100 cc. of blood lost.

Time.	No.	Ether.	Lactic acid.	pH	Total CO_2 as drawn.		CO_2 of equilibrated sample.			Oxygen chemically bound.	
					Content.	Tension.	Content.	Tension.	Content at 38 mm.	Content.	Capacity.
a.m.		*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*
8.50	1		Lost.	7.43	43.2	35.7	42.7	34.3	44.5	19.8	20.6
9.04	2	130	41.5	7.38	46.5	42.9	42.8	33.2	44.0	17.1	19.7
9.20	3	158	51.5	7.36	39.2	37.0	39.8	39.2	39.5	19.5	17.8(?)
10.07	4	189	69.2	7.20	32.8	44.4	27.1	33.2	29.5	19.2	21.1
10.45	5	213	75.2	7.14	34.1	52.1	29.8	36.1	30.5	20.9	21.2

Experiment IV.

Very Deep Anesthesia.

Male dog. Weight 10.64 kilos. Initial sample 9.05 a.m. Ether started 9.10 a.m. Cone placed over nose of dog till respiration stimulated. Ether then poured on mask. Unconscious in ½ minute, when mask was loosened to increase ventilation. Tracheotomy. Dog attached to ether apparatus. Anesthesia deep after the first 20 minutes of experiment. Respiration paralyzed in 4 hours and 20 minutes. 150 cc. of blood lost.

Time.	No.	Ether.	Lactic acid.	pH	Total CO_2 as drawn.		CO_2 of equilibrated sample.			Oxygen chemically bound.	
					Content.	Tension.	Content.	Tension.	Content at 38 mm.	Content.	Capacity.
a.m.		*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*
9.05	1		13.1	7.30	36.2	39.5	33.3	35.8	34.2	18.3	21.3
9.14	2	138	16.2	7.29	36.8	41.3	34.7	37.9	34.2	19.8	21.4
9.35	3	160	14.4	7.32	31.5	33.1	33.4	36.0	34.2	19.9	21.1
10.17	4	160	38.8	7.18	26.2	36.9	25.8	36.0	27.0	21.1	21.0
11.00	5	162	48.2	7.12	29.7	47.4	25.5	35.7	26.2	20.1	22.1
11.45	6	160	53.6	7.08	28.7	44.9	22.9	35.9	23.7	20.1	21.1
p.m.											
12.50	7		62.3	7.05	29.3	54.0	22.2	37.8	22.2	20.3	21.2
1.30	8	160	76.2								

Experiment V.

Moderate Anesthesia for 2 Hours and Recovery.

Dog I. Weight 11.2 kilos. Initial sample of blood 9.20 a.m. Cone placed over nose 9.25 a.m. After 1 minute ether poured on. Fully unconscious at the end of the 2nd minute when the cone was replaced by several layers of gauze, soaked with ether. Marked but brief struggling. Ether off at 11.35 a.m. Vomiting in 15 minutes. Lasted only short time. Total loss of blood 150 cc.

Time.	No.	Ether.	Lactic acid.	pH	Total CO_2 as drawn.		CO_2 of equilibrated sample.			Oxygen chemically bound.	
					Content.	Tension.	Content.	Tension.	Content at 38 mm.	Content.	Capacity.
a.m.		*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*
9.20	1		16	7.33	37.9	38.4	25.8(?)	33.5	37.0	19.3	20.6
10.05	2	122	46	7.21	34.2	45.1	31.7	35.7	32.5	19.8	20.5
11.30	3	128	53	7.09	32.4	55.0	27.4	34.2	28.0	18.6	21.7
p.m.											
12.30	4	28	32	7.24	27.5	33.6	28.8	35.2	29.0	19.2	19.3
2.30	5		35	7.23	32.9	41.1	31.0	34.0	31.8	17.5	18.9
24 hrs. later.	6		19	7.27	36.3	41.7	35.0	38.1	34.5	17.3	18.4
5 hrs. later.	7		16	7.29	46.9	52.0	40.5	38.7	40.0	18.0	18.4
20 hrs. later.	8		17	7.31	38.2	40.3	37.4	38.4	37.0	17.9	18.4

Experiment VI.

Effect of Low Oxygen Tension. Recovery Followed.

Dog I. Second experiment. Weight 11.6 kilos. Initial bleeding 9.10 a.m. Cone placed over nose of dog till respiration stimulated. 9.15 a.m. Ether started. 9.20 a.m. Respiration stopped. Artificial respiration. Mask used throughout experiment. Tendency for respiration to be stimulated due to increased CO_2. Ether stopped at 11.20 a.m. Total loss of blood 150 cc.

Time.	No.	Ether.	Lactic acid.	pH	Total CO_2 as drawn.		CO_2 of equilibrated sample.			Oxygen chemically bound.	
					Content.	Tension.	Content.	Tension.	Content at 38 mm.	Content.	Capacity.
a.m.		*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*
9.10	1		21.6	7.33	41.8	42.9	39.1	37.2	39.4	15.3	18.0
11.20	2	126	63.5	7.08	38.3	65.5	24.6	38.7	24.0	8.1	19.8
p.m.											
1.15	3			7.08	33.1	57.5	23.1	35.7	24.0	15.9	19.6
2.45	4		54.0	7.18	32.2	45.4	27.8	37.6	28.0	15.8	17.1
5.15	5		21.5	7.26	39.3	46.3	36.3	37.2	35.7	16.5	18.2
Next day.											
a.m.											
9.30	6		24.5	7.28	39.8	45.4	35.6	37.2	35.2	16.0	17.9
p.m.											
4.20	7		31.0	7.40	29.5	26.2	37.9	36.4	38.0	18.9	18.6

Experiment VII.

Effect of Low Oxygen Tension. Recovery Followed.

Dog I. Third experiment. Male. Weight 11.3 kilos. Initial sample 8.50 a.m. Cone placed tightly over nose till respiration stimulated. Ether started 9.05 a.m. ½ minute of struggling. Mask was used throughout the experiment. Even though it fitted loosely over the nose the O_2 unsaturation of the blood shows reduced O_2 tension. 11.05 a.m. Ether stopped. Loss of blood during 1st day of experiment 180 cc. 2nd day 15 cc. Sample 10 taken with considerable struggling.

Time.	No.	Ether.	Lactic acid.	pH	Total CO_2 as drawn.		CO_2 of equilibrated sample.			Oxygen chemically bound.	
					Content.	Tension.	Content.	Tension.	Content at 38 mm.	Content.	Capacity.
a.m.		*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*
8.50	1		18.5	7.31	38.6	41.4	33.7	35.3	36.0	15.7	19.1
9.10	2	120	24.5	7.22	38.4	49.7	33.4	37.3	33.2	15.9	19.0
9.20	3		27.5	7.14	40.5	59.5	31.1	35.3	32.1	15.7	19.2
9.39	4	125	33.0	7.10	41.4	68.2	28.0	36.1	29.8	9.9	19.6
10.08	5		65.0	7.05	35.6	65.3	24.2	34.3	25.5	11.2	20.0
10.35	6		58.0	7.05	28.3	51.8	22.9	34.9	23.5	16.3	20.2
11.05	7	120	82.5	7.04	26.7	50.2	22.4	35.7	23.0	16.6	19.6
p.m.											
12.05	8		78.5	7.06	25.9	46.7	22.6	37.8	22.1	19.9	19.7
4.00	9		22.0	7.34	34.5	34.6	36.6	37.9	36.6	20.1	20.7
Next day.											
a.m.											
10.00	10		35.5	7.31	40.0	42.8	35.8	37.5	36.0	15.6	18.5

Experiment VIII.

Dog. Male. Weight 12.2 kilos. Initial sample 8.50 a.m. Ether started 9.05 a.m. Tracheotomy. Attached to ether apparatus 9.10 a.m. Concentration kept constant. Respiration depressed. Animal dies of respiratory failure 10.30 a.m. Loss of blood 70 cc.

Time.	No.	Ether.	Lactic acid.	pH	Total CO_2 as drawn.		CO_2 of equilibrated sample.			Oxygen chemically bound.	
					Content.	Tension.	Content.	Tension.	Content at 38 mm.	Content.	Capacity.
a.m.		*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*
8.50	1		27.5	7.45	38.2	33.0	39.9	39.6	39.5	26.4	29.6
9.35	2	183	45.3	7.10	44.9	74.8	32.2	32.6	34.0	18.7	30.3
10.05	3	189	58.0	7.08	36.1	62.4	26.3	33.8	27.5	18.8	29.9
10.30	Dead.										

Experiment IX.

Mild Anesthesia. Recovery Followed.

Dog II. Male. Weight 14.3 kilos. Initial sample 8.40 a.m. Mask placed over nose till respiration stimulated. Ether started, 8.50 a.m., without more than ½ minute struggling. Mask replaced by gauze, drop method of administration continued. Salivation marked. Animal choked before Sample 2 was taken. Ether stopped 11.00 a.m. During recovery vomited frequently; due to swallowing of ether with saliva. Vomitus consisted largely of saliva. Loss of blood 150 cc.

Time.	No.	Ether.	Lactic acid.	pH	Total CO_2 as drawn.		CO_2 of equilibrated sample.			Oxygen chemically bound.	
					Content.	Tension.	Content.	Tension.	Content at 35 mm.	Content.	Capacity.
a.m.		*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*
8.40	1		25.0	7.38	34.0	31.3	33.9	30.5	36.0	21.6	22.7
9.20	2	100	26.6	7.36	33.9	32.0	29.9	27.3	35.5	22.6	22.4
9.50	3	115	56.8	7.33	29.2	30.0	31.1	35.8	31.0	22.4	22.1
10.50	4	110	66.9	7.22	24.1	31.1	24.4	31.0	25.5	21.5	23.2
p.m.											
12.00	5		61.0	7.20	28.7	38.8	26.6	33.5	27.4	21.1	22.2
2.00	6		33.7	7.45	32.3	25.6	35.5	31.6	36.0	20.4	20.4
4.00	7		18.3	7.38	36.9	34.3	35.6	31.6	36.0	18.4	19.7
Next day.											
a.m.											
9.00	8		23.8	7.37	34.4	32.4	36.7	37.4	35.5	20.1	20.1

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THE DIFFERENTIAL EXTRACTION AND PRECIPITATION OF THE SOLUBLE PROTEINS OF MUSCLE, WITH DATA ON THE CONCENTRATION OF PROTEINS IN THE MUSCLE OF THE CALF, COW, AND RABBIT.

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In 1917, Dr. Theobald Smith called our attention to the practical problem of obtaining a clear bacteriological medium from extracts of the muscles of very young calves. Media prepared from such extracts became opalescent during sterilization and remained so. The immediate difficulty was overcome by a slight change in the reaction of the extract, acid, before the coagulation of the soluble proteins. At that time various experiments were made to determine the cause of the turbidity. One of the problems considered was that of a difference in the kind or character of the proteins of the muscle. The existing procedures for the separation and estimation of muscle proteins presented many variables with regard to the technique of extraction and of coagulation. In addition to the technical errors there were the unknown factors of "coagulation" and the development of acidity which take place after the death of an animal.

Our early experiments dealt with the use of salts of various kinds, formerly used for extraction, with the addition of mixtures of monosodium and disodium phosphate having a hydrion concentration of pH 7.0. The latter mixture was adopted in an attempt to neutralize the acids as they are formed or to dissolve proteins which might have been precipitated by acid. The temperature of coagulation was used as the method of differentiating the proteins. The confusion in the literature with regard to the solvent required for the extraction of muscle led

us to make some comparative experiments on the concentration of salt required for the extraction of muscle proteins. We found a point of maximum extraction for magnesium sulfate and one for sodium sulfate. In the meantime the relative values of different salts for the purpose of precipitating the proteins of blood and the critical zones which occur in the curve of precipitation had been determined (1, 2). These findings offered a method of attack on the problem which has resulted in a clarification of the differences between various investigators and a method for the comparative study of the composition of muscle. The most characteristic protein of muscle is very labile. The procedure of extracting at the same time a series of samples of muscle with definite concentrations of salt offers a means of arriving at the composition of muscle which does not carry the possibility of an error from a change in the labile protein fraction of muscle—an error which is likely to occur in the fractionation of a single extract.

Procedures.—The procedures adopted in studying the muscle of calves were in part dictated by necessity, while others were selected because they seemed adapted to give the best results. Some of the more important reasons for the procedures are as follows: It was desirable to extract the muscle as soon as possible after death to minimize the changes which occur in rigor mortis. It was not possible to wash out the blood from the blood vessels by perfusion. For a preliminary study of the composition of the muscle of calves and a survey of procedure, the muscles were removed from an animal as soon as possible after the animal was killed, ground in a meat grinder, definite quantities of the hash, 5 gm., weighed and introduced into a given quantity of a salt solution of a concentration such that the desired concentration would be attained when the water of the muscle was taken into consideration. All muscle samples were in the salt solution in from $\frac{1}{2}$ to 1 hour after the death of the animal according to the number of determinations which were to be made.

The basis for the adoption, or justification for the use of certain of the procedures, is presented in the following discussion.

Distribution of Salt in the Salt Solution-Muscle Mixture.—The water of muscle tissue may be considered, for all practical purposes, as free water such that upon the addition of a solution whose

concentration is higher than the desired concentration, by the amount of salt necessary to bring the water of the muscle to the desired concentration, it will dilute the concentrated solution. The effect of muscle-water on the concentration of salt in the ultimate solution becomes less as the ratio of the volume of solution to the quantity of meat is increased—for small quantities of salt solution the error may be considerable.[1] Data obtained from the analysis of muscle extracts show that for larger quantities of meat in proportion to the solution than are used in this work the desired concentration is attained or is slightly less. The concordance of the results obtained for non-protein nitrogen from extracts made with different concentrations of salt solution representing different amounts of protein extracted supports the evidence obtained by analysis of the concentration of salt.

The volume of water associated with the muscle has been assumed to be 4 cc. for 5 gm. of muscle, which corresponds very closely with the data on the moisture content of the muscle and is within the limits of experimental error when 50 or 100 cc. of solution are used.

Distribution of Protein between the Muscle Tissues and the Salt Solution.—It was found by means of successive extractions of a given sample of muscle that the soluble proteins are distributed uniformly between the muscle and the solutions. Portions of 5 gm. each of muscle were distributed into 25, 50, or 100 cc. portions of different salt solutions of various concentrations and after 1 to 2 hours, during which time the tubes were inverted occasionally, the supernatant liquid was filtered off, measured, and samples were taken for analysis. To the residue and associated liquid, which varied in amount, a quantity of salt solution of the same concentration equal in amount to the liquid removed was added. After 1 or 2 hours the resulting solutions

[1] That such an error may occur in the precipitation of protein is shown by the analyses of Spiro (3), of Sörensen and Höyrup (4), and by data which we have obtained. These results indicate that where the volume of precipitate is large in relation to the total volume of solution and precipitate there is a concentration of salt in the supernatant liquid and a greater precipitation of protein than would occur if such a concentration had not taken place.

were again filtered, measured, and analyzed. This process was repeated until five extractions had been made. From the data obtained calculations were made of the amount of nitrogen which should have been obtained in the various extractions after the first on the basis that the protein nitrogen found in each case represents that amount which would be obtained upon dilution of the liquid remaining with the muscle residue in the previous extraction. Typical results of such experiments are contained in Table I. It is evident (*a*) that there is not an increased extraction of protein upon repeated extraction, and (*b*) that extraction is essentially complete by the fourth extraction—data for the fifth extraction are included in only one case since the quan-

TABLE I.

Repeated Extraction of the Same Sample of Muscle with Salt Solution. Grams of Nitrogen per 100 Cc. of Extract.

No. of extraction.	(K)PO_4* 0.525—25 cc.		H_2O—25 cc.		Li_2SO_4 0.6—50 cc.		(K)PO_4 0.225—50 cc.	
	Found.	Calculated.	Found.	Calculated.	Found.	Calculated.	Found.	Calculated.
1	0.265		0.133		0.084		0.222	
2	0.091	0.094	0.038	0.044	0.022	0.019	0.140	0.135
3	0.028	0.033	0.015	0.014	0.008	0.008	0.064	0.065
4	0.009	0.011	0.006	0.006	0.004	0.004	0.027	0.032
5							0.002	0.001

* (K)PO_4 is used to designate a mixture of monopotassium and dipotassium phosphates in the proportion of 1:2.

tity of nitrogen present in the other samples was less than that represented by the experimental error of the Kjeldahl determinations. If the concentration of salt be changed, with either a higher or a lower concentration of salt, after the first series is completely extracted no more protein is removed, due apparently to the fact that the protein becomes insoluble; it has been possible in some cases to extract nitrogen in the case of a lower concentration after a higher concentration, but very seldom when the fraction is the most labile protein fraction.

Adequacy of the Sampling for Individual Determinations.—We have obtained repeated evidence in the reproducibility of results that the sampling of muscle is adequate. With muscle tissue, as with serum proteins, a given concentration of salt solution

will give equal extraction under like conditions. Reproducibility of results is, therefore, not evidence of the presence or absence of a protein fraction. Duplicate or triplicate extractions usually agree. Considering the technique employed and the readiness with which certain of the proteins become insoluble the results are very good. We feel that the error with regard to the original muscle tissue is much less in the case of multiple extractions than it would be by fractioning a solution obtained from a single sample of a larger quantity of muscle.

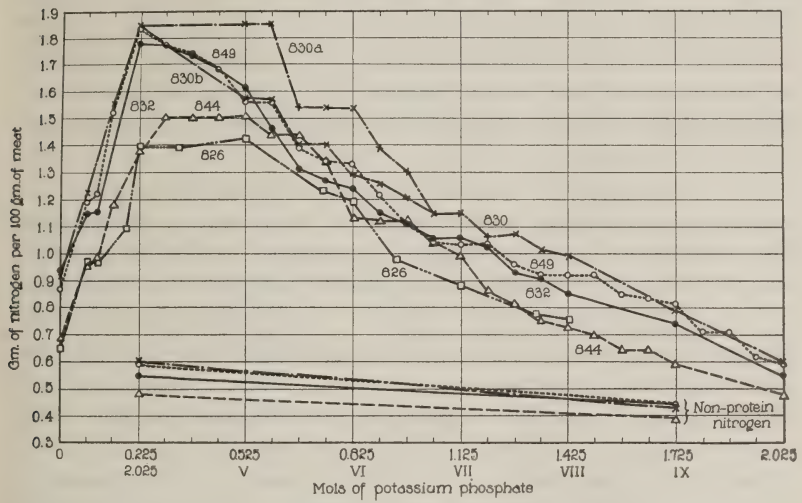

CHART 1. Showing the effect of increasing concentrations of a mixture of monopotassium and dipotassium phosphates 1:2 on the quantity of protein extracted from muscle. Results are expressed as grams of nitrogen in 100 gm. of fresh muscle.

Differential Extraction of the Proteins of Muscle.—The differential extraction of the proteins of muscle rests on the observations (*a*) that beginning with water (which includes the salts in the muscle) there is an increasing extraction of muscle protein up to a certain zone after which the quantity obtained is less; (*b*) that there are critical zones in the curve of decreasing extraction, after the maximum is reached, which are assumed to represent different fractions of muscle proteins (Chart 1). There is a possible fallacy in the last assumption, which applies also

to the proteins of blood plasma; it is the possibility that the critical zones represent a step in periodical changes in the precipitating capacity of salts or in the physical character of the proteins. Certain of the critical zones in muscle, as with the plasma proteins, agree fairly well with the work of previous investigators (5, 6). In the case of muscle, however, there is not the agreement between investigators which exists in the case of the blood proteins. The evidence of Halliburton (5) and of von Fürth (6) can be correlated with our data when interpreted in the light of the evidence presented here (see later discussion of the evidence). Work on the protein fractions of muscle obtained is required before they can finally be accepted as representing true protein fractions; such work is much more difficult with the first, and probably most characteristic fraction, because of its lability.

Data bearing on the quantity of protein extracted by different concentrations of salt are summarized in Chart 1. Additional data with regard to the maximum extraction have been obtained in numerous other analyses of muscle in which the variations in concentration were not so small. Data relating to Samples 826 and 844 (calf muscle) and 832 and 849 (rabbit muscle) are the averages of duplicate determinations. The data for Samples 830 *a* and 830 *b* (rabbit muscle) represent averages with the exception of the portions of the curve marked "*a*" and "*b*." These latter deviations are presented because they represent changes which may take place in muscle within a relatively short time—the samples for analysis were weighed out in sequence for the whole series, No. 830 *a* first, then various other determinations, and finally No. 830 *b* last.

It is evident that the maximum extraction of protein from muscle occurs between 0.225 and 0.525 molar solutions of potassium phosphate (KH_2PO_4, 1 part, to K_2HPO_4, 2 parts). There is evidence of critical zones at approximately 0.525, 0.825, 1.125, 1.425, 1.725, and 2.025 molar potassium phosphate. The data might be interpreted as indicating critical points slightly removed from those given. Our interpretation is influenced by the facts that (*a*) a 0.225 molar solution of potassium phosphate is directly related to the concentration of salt required for the precipitation of the protein "fractions" of blood. Such a solution is *less* than

the concentration required for the precipitation of fibrinogen (1.125 mols) by three times the increment for this salt (2) as found for the various protein fractions of blood.[2] (*b*) Within the range of its solvent or precipitating action the different salts produce essentially the same effects at concentrations which are related to those for blood proteins.

The maximum extraction does not always occur at 0.225 molar potassium phosphate; it has always occurred at this concentration for rabbit muscle, but may occur with a 0.525 molar solution. The reason for this difference has not been found. We are inclined, at present, to ascribe the variations to the lability of the protein extracted at 0.225 mol.

Relative Solvent and Precipitating Capacity of Salts.—In determining the relative solvent and precipitating capacity of different salts for muscle proteins the assumption was made that the conditions which hold for the precipitation of plasma proteins would hold for the muscle proteins. To aid in the presentation of the subject the volume-molar concentrations of various salts required for the equal precipitation of the proteins of blood plasma, Fractions I to IX, and the probable additional concentrations (*A* to *D*) which apply to muscle protein are presented in Table II.

A study of the different salts is complicated in two ways. (*a*) The necessity for working with very fresh material which requires that all extractions be made simultaneously on the same muscle sample—this results in a large number of determinations. There is not an opportunity to check an obviously erroneous result since the material has changed in the meantime. (*b*) The rapidity with which the proteins extracted by the lower concentrations of salt becomes modified in the presence of relatively low hydrion concentrations. This is particularly true in

[2] Attention is called to the concentrations 0.075, 0.375, 0.675, 0.975, 1.275, 1.575, and 1.875 mols which are 0.15 mol, *i.e.* one-half the increment, removed from the selected concentrations. These concentrations might almost as well have been selected but for the results obtained at 0.225 mol. It is possible that changes in the rate of precipitation or extraction occur at 0.15 mol instead of 0.3 mol as has been developed for the blood proteins and that the concentration of blood proteins is not sufficiently great to give evidence of the intermediate values.

TABLE II.

Volume-Molar Concentrations of Various Salts Required for the Equal Precipitation of the Proteins of Blood Plasma (Fractions I to IX) with the Probable Concentrations for Muscle Proteins (Fractions A to D).

Salt.	Increment.					Globulin fractions of blood.				Albumin fractions of blood.				
		D	C	B	A	I	II	III	IV	V	VI	VII	VIII	IX (total protein).
Na_2SO_4	0.25			0.25	0.50	0.75	1.00	1.25	1.50	1.75	2.00	2.25	2.50	2.75
$(NH_4)_2SO_4$	0.25	0.25	0.50	0.75	1.00	1.25	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25
$MgSO_4$	0.375		0.225	0.50	0.875	1.25	1.625	2.00	2.375	2.750				
Li_2SO_4	0.40			0.20	0.60	1.00	1.40	1.80	2.20	2.60				
$\frac{KH_2PO_4}{K_2HPO_4}$: $\frac{1}{2}$	0.30		0.225	0.525	0.825	1.125	1.425	1.725	2.025					
$\frac{NaH_2PO_4}{Na_2HPO_4}$: $\frac{1}{2}$	0.30			0.30	0.60	0.90	1.20	1.50	1.80					
NaCl	1.25			1.25	2.50	3.75	5.00							

the case of salts having a low buffer value, which includes practically all the salts except the mixtures of the phosphates. It is not a difficult matter to obtain duplicate determinations with equal concentrations of the same salt. When different salts are compared on the basis of the data in Table II the results may or may not show the same relations on two different samples of muscle. The fact that when there is agreement it is usually very good and that the marked deviations occur in Fractions *A* to *D* (Table II) has led us to the conclusion that the variability of results is to be laid particularly to changes in the muscle protein. The data in Table III are presented to show the relation

TABLE III.

*Nitrogen Extracted from Muscle by Various Concentrations of Different Salts. Results Are Expressed as Grams of Nitrogen in 100 Gm. of Muscle.**

	Volume-molar concentration of salt.						
$(K)PO_4$	0.225	0.525	0.825	1.125	1.425	1.725	2.025
$MgSO_4$	0.225	0.50	0.875	1.25	1.625	2.00	2.375
Li_2SO_4		0.20	0.60	1.00	1.40	1.80	2.20
	Nitrogen extracted.						
$(K)PO_4$	1.58	1.52	1.38	1.01	0.85	0.76	0.58
$MgSO_4$	1.59	1.53	1.35	1.02	0.92	0.75	0.58
Li_2SO_4		1.53	1.35	1.02	0.87	0.75	0.59

* Total nitrogen, 3.18 per cent; moisture, 76.4 per cent; H_2O extract, 0.69 gm. of N per 100 gm. of muscle.

between a mixture of the potassium phosphates, magnesium sulfate, and lithium sulfate. These three solvents usually agree in their solvent and precipitating action when used on muscle.

An attempt was made to determine for each of the salts the concentration at which it shows the maximum solvent effect. It was possible that such salts as sodium sulfate, lithium sulfate, sodium chloride, and a mixture of sodium phosphates would extract the maximum amount of protein at concentrations indicated as *B* (Table II) and that all lower concentrations would dissolve less protein from muscle. Magnesium sulfate and a mixture of the potassium phosphates, on the other hand would have their maximum solvent action at lower concentrations, *C*,

while ammonium sulfate and chloride would give the maximum effect at concentration *D*. This is, of course, comparing the salts on the basis of their relation to the concentrations required for the precipitation of the proteins of blood and to the increment found for the fractions in blood plasma. For the reasons given above the experimental data are not clear-cut in all cases. There is a further complication in the case of the muscle of the calf and cow in that the maximum extraction does not always occur at 0.225 molar potassium phosphate, which we have used as a reference concentration, but at a higher concentration, 0.525 mol; rabbit muscle is much more satisfactory. The only definite conclusion which we have reached is that the maximum extraction is attained at concentrations *B* or *C*. Ammonium sulfate (7) does not give a greater extraction at concentration *D* than at *C*. The maximum extraction for ammonium chloride is at 1.25 mols, or five times the concentration for ammonium sulfate, which is in agreement with the relation between chlorides and sulfates previously found. This concentration for ammonium chloride is also that found by previous investigators as giving the maximum extraction of muscle protein, 15 per cent. Sodium chloride has given the maximum extraction at 1.25 mols.

When the data are considered on the basis of the first concentration, irrespective of the increments, there is a slight indication that the maximum extraction is attained at approximately 0.2 to 0.25 mol for the sulfates and phosphates and five times the concentration for the chlorides. Precipitation, *i.e.* a lower extraction, does not begin in the absence of postmortem changes until after concentration *B* (Table II).

Use of Phosphate Mixtures for the Extraction and Precipitation of Muscle Proteins.—A mixture of the KH_2PO_4 and K_2HPO_4 in the ratio of 1:2, which gives a hydrion concentration of 7.0 when sufficiently diluted,[3] has been found to be the most satisfactory for use in estimating the distribution of the proteins of muscle. A similar mixture of the sodium phosphates has only

[3] Dr. A. L. Marshall, of Princeton University, has kindly measured the hydrion concentration of a 2.25 volume-molar solution of such a mixture. His results, measured against saturated calomel electrode, are: undiluted, 0.4336 volt, pH 7.39; diluted with an equal volume of water, 0.4137 volt, pH 7.05; diluted with 3 volumes of water, 0.4106 volt, pH 7.0.

a limited use at room temperature because of the relative insolubility of the basic phosphate. The mixture of potassium phosphates extracts as much protein as any other neutral salt examined. It has the advantage that the proteins do not so readily become "denatured" with the precipitation of a part or all the extracted protein, such as occurs more or less regularly with sodium chloride and to a less extent with lithium sulfate, magnesium sulfate, ammonium sulfate, or ammonium chloride. There is no reason to suppose that what occurs in the extracts of these salts after filtration may not and probably does in some cases occur in the process of extraction before filtration. Potassium phosphate extracts of muscle at concentrations *B* and *C* (Table II) may undergo a gradual change with the separation of a small amount of finely divided precipitate in the course of 12 to 24 hours. The amount of precipitate is not commensurate with the quantity of protein represented by these fractions. More often the extracts become increasingly opalescent in reflected light without the formation of a precipitate. The matter of the transformation of muscle proteins will be discussed later.

Effect of the Cation in the Solution and Precipitation of Muscle Protein and of the Variation in Hydrion Concentration.—A change in the proportion of phosphate ion does not influence the quantity of protein extracted provided the concentration of the cation is kept constant and at hydrion concentrations less and somewhat removed from the isoelectric point of the protein. The effect of the variation in the concentration of the phosphate ion, and hydrion concentration, while keeping the potassium ion concentration the same, is illustrated in Table IV. There are certain determinations in the table as indicated, which are undoubtedly incorrect. Because of the number of determinations it was only possible to make one extraction at each concentration of salt and, as already explained, it is not possible to repeat a determination. The data in any vertical column were obtained with solutions having the same concentration of potassium ion and a variable amount of phosphate ion. A horizontal row contains data on solutions having approximately the same hydrion concentration.

Variations in Hydrion Concentration.—Some of the effects of changes in the hydrion concentrations are evident in Table IV.

By using a mixture of the sodium phosphates for the acid solutions it is possible to extend the series in the direction of higher concentrations of salt, but no important additional information has been obtained. When the data are examined with relation to those obtained at a mixture of KH_2PO_4:K_2HPO_4, 1:2, there is not an appreciable decrease in protein extraction, *i.e.* precipitation, until a phosphate ratio of 1:1 (pH 6.7) is attained. Between 1:1 and 2:1 (pH 6.4) there is a decrease in extraction

TABLE IV.

Extraction of Muscle with Various Mixtures of Potassium Phosphate Having the Same Potassium Ion Concentration (in Any Vertical Column) as That of a Mixture of KH_2PO_4:K_2HPO_4 of 1:2. Results Are Expressed as Grams of Nitrogen per 100 Gm. of Muscle.

Concentration of PO_4 at a ratio $\frac{KH_2PO_4}{K_2HPO_4}$: $\frac{1}{2}$*		C	B	A	I	II*
	0.109	0.225	0.525	0.825	1.125	1.425
Ratio $\frac{KH_2PO_4}{K_2HPO_4}$						
1:0	0.67	0.67	0.68	0.57		
16:1	0.83	0.80	0.63	0.70		
8:1	0.85	0.94	0.90	0.83		
4:1	0.85	0.98	1.01	0.99		
2:1	0.85	1.12	1.29	1.16	0.82	0.73
1:1	0.92	1.19†	1.44	1.25†	0.81	0.74
1:2	**0.97**	**1.39**	**1.44**	**1.19**	**0.88**	**0.74**
1:8	0.98	1.43	1.47	1.17	0.95‡	0.76
1:32	0.98	1.45	1.38	1.16	0.88	0.77
0:1	0.97	1.48	1.50	1.11	0.86	0.76

* The letters and numbers in this row are the same as those in Table II.

† This value has agreed with that at 1:2 in three other cases.

‡ This value has agreed with that at 1:2 in two other cases.

at potassium ion concentrations *B* and *C*, but not at *A*, or at the equivalent of 0.109 molar potassium phosphate. At a ratio of 4:1 the quantity of protein extracted is essentially the same for concentrations *A*, *B*, or *C*. It appears that the first protein fraction of muscle is precipitated at a hydrion concentration between 6.7 and 6.0, which is in general agreement with the data of Collip (8) on the isoelectric point of muscle protein. There does not appear to be an effect of hydrion concentration

for Fractions I and II up to phosphate ratios of 2:1. In solutions more alkaline than pH 7.0 the hydrion concentration does not have an appreciable effect, even in pure dipotassium phosphate.

The results obtained with pure monopotassium phosphate are of considerable interest. The *water-clear* extracts, essentially free from coloring matter, became rapidly turbid upon standing. The turbidity, and ultimate precipitation, developed most rapidly at high concentrations of phosphate, at least they were entirely absent at the lowest concentration. The effect is apparently one of a change in the protein molecule which has a time factor and is related to the concentration of the salt.[4]

Extracts of muscle made at hydrion concentrations less than pH 6.7 and particularly pH 7.0 and above (alkaline solution) remain clear or do not develop a slight turbidity on standing so rapidly as extracts made at higher hydrion concentrations. It is our opinion that the transformations in muscle extracts observed by von Fürth (9) are related to changes in hydrion concentration rather than to an actual change from one type of protein to another. The acidity of unbuffered extracts, such as water, sodium chloride, and lithium and magnesium sulfates, is usually high enough, at least, to be on the verge of precipitation. Even at 37°C. phosphate extracts of muscle will remain clear—no precipitate—for a week or more. It is not to be implied that precipitates do not form in the phosphate extracts at times at 37°C., particularly in the low concentrations of salt. The evidence is more in the direction of a denaturation of the proteins rather than a transformation of one protein into another. In one case an extract of muscle with a 0.525 molar solution of potassium phosphate did not show any indication of precipitation when held at 37°C. for 2 weeks, nor for 3 additional weeks at room temperature. This solution did show evidence of a change for the solution became decidedly opalescent, almost milky in

[4] In experiments with sodium phosphate the turbidity at Concentrations I, II, III, IV were less in the order named. There was no coagulable nitrogen in IV. At a phosphate ratio of 32:1 the maximum turbidity occurred in concentrations *B* and *C*, at a ratio of 16:1 only a trace of turbidity at *B*, while at a ratio of 8:1 there was no increase in turbidity on standing.

appearance. On the other hand, coagulation with heat took place at 47–50°C. just as did a fresh extract at the same concentration or a fresh extract made with sodium chloride or lithium sulfate.

Heat Coagulation of Muscle Extracts.—Previous workers on muscle have relied particularly on the temperature of coagulation of the various protein fractions or extracts of muscle as the means of identification of the muscle proteins. Most of our early work was with this procedure and from time to time extracts have been coagulated in order to correlate this work with the previous work.

The data obtained from the coagulation of a series of extractions made with potassium phosphate mixtures of various concentrations are the most important of the work in this direction. The solutions remaining from the series of extractions shown in Table IV were heated in a water bath, which was kept within 0.5°C., at various temperatures. These extracts had stood at room temperature for 18 hours before they were coagulated, which would have permitted the formation of von Fürth's soluble myogen fibrin to have taken place if this occurred in the phosphate solutions. Precipitations had not developed in the solution during this time nor had the opalescence in the lower concentrations of salt perceptibly increased with the following exceptions: (*a*) all the extracts in KH_2PO_4 contained precipitates as indicated above, and (*b*) extracts at 0.109 mol between the salt ratios of 16:1 to 1:1 were slightly turbid.

Data Showing the Temperature of Coagulation of Potassium Phosphate Solutions of Various Hydrion and Salt Concentrations.[5]

Water.

40°C. Slight opalescence.

50°C. Flocculation.

0.109 molar phosphate solution.

40°C. for 30 min. Increased turbidity between 1:0 and 8:1, but not flocculation. No change between 4:1 and 0:1.

40–50°C. for 20 min. Coagulation in solutions 1:0 to 2:1. Solution 1:1 turbid only. No change between 2:1 and 0:1.

50–63°C. Solutions of 1:1 to 1:8 coagulated at 55°C. Solutions 1:32 to 0:1 coagulated at 63°C.

[5] The ratios refer to the relative proportions of KH_2PO_4 and K_2HPO_4.

0.225 molar phosphate solution.

40°C. for 30 min. The turbidity increased for solutions 1:0 to 8:1, but no coagulation.

40–50°C. Coagulation of solutions 1:0 to 1:1 (slight turbidity in 1:1).

50–63°C. Solutions 1:1 to 1:8 were turbid at 58°C. and gave gelatinous precipitates at 63°C. Solution 0:1 gave a finely divided precipitate at 63°C.

0.525 to 0.825 molar phosphate solutions.

40°C. No appreciable change in solutions 1:0 to 16:1. Slight opalescence for solutions 8:1 to 4:1. Remainder unchanged.

40–50°C. Coagulation in solutions 8:1 to 1:1. (Represented at 4:1 to 1:1 by an increased turbidity, but not flocculation.)

50–56°C. Coagulation in solutions 1:2 to 0:1.

1.125 and 1.425 molar phosphate solutions. (Solutions 1:0 to 4:1 not tested.)

40°C. No change.

40–50°C. After 30 min. at 50°C., fine flocculent precipitate.

60°C. A second precipitate heavier than that at 50°C.

Various experiments have been made with purified fractions of muscle protein, but they have not led to any important additional information.

The obvious conclusion from the heat coagulation of muscle extracts presented above is the well known fact that coagulation takes place most readily in acid solutions. The same relation holds with regard to standing at room temperature. At 38°C. protein is precipitated from solutions of salts of low buffer value. Highly buffered solutions neutralize the acid formed in muscle and thus prevent the changes due to an increased hydrion concentration.

These observations have a particular bearing upon the identity and significance of von Fürth's soluble myogen fibrin. Instead of considering soluble myogen fibrin as a definite transition protein between his myogen and myosin it may be considered as an evidence of denaturation or change under the influence of the hydrion concentration and of the salt concentration.

Von Fürth (10) has questioned recently his old interpretation of his original observations on soluble myogen fibrin. He calls attention to the probability that the gradual aggregation of myogen (myosinogen) molecules is the explanation of the appearance of a precipitate in a muscle extract upon standing. The aggregation is hastened even at low temperatures; which would explain the low coagulation point of his soluble myogen

fibrin. The value of the temperature of coagulation as a means of identifying proteins is also questioned by von Fürth as has been done by others in the past.

DISCUSSION.

The detailed discussion of the characteristics and properties of the proteins of muscle presented by Halliburton (5) and by von Fürth (9, 10) makes it unnecessary to present the general facts with regard to our knowledge of this subject. Since the original papers of these two men, Bottazzi and Quagliariello (11) have introduced a new element into the discussion of the soluble proteins of muscle, the ultramicroscopic granules which they assume to be the myosin of von Fürth or the paramyosinogen of Halliburton.

Before considering our data in its relation to the commonly accepted evidence with regard to muscle proteins it is necessary to discuss the significance of the critical zones in the curve of precipitation of naturally occurring protein mixtures and the regularity of occurrence of these zones. The presence of critical zones in the curve of precipitation of the proteins of blood serum was first presented for sodium sulfate (1). That such zones probably existed was to be concluded from work on the precipitation limits of protein with ammonium sulfate; they had not been demonstrated, however, because the dilution of the protein solutions had not been sufficiently great. Subsequent work (2) showed that the additional concentration of salt required for the precipitation of each succeeding fraction of blood plasma, after the first fraction had been precipitated, was the same, this difference was called the increment of salt. The comparison of various salts whose solubility was sufficiently great to precipitate more than one of the fractions showed that there was a characteristic increment for each salt, some of the results have been summarized in Table II.

As the result of the study of the extraction and precipitation of the proteins of muscle it has not been necessary to modify the conclusions which resulted from the study of the proteins of blood plasma.[6] The concentrations of salt required for the

[6] The evidence presented in Chart 1 on the extraction of the proteins of muscle suggests the possibility that there may be a smaller increment equal to one-half that found for the proteins of blood.

precipitation of the protein fractions of blood are related to the concentrations for the maximum extraction of the proteins of muscle and the critical zones for muscle proteins as multiples of the increment of salt required for the blood proteins. Thus for the mixture of monopotassium and dipotassium phosphates 1:2, the maximum extraction from rabbit muscle occurs at 3×0.3 mol *less* than the concentration required to precipitate fibrinogen; the increment for the blood proteins is 0.3 mol. We have amply verified the fact that any concentration of salt will extract or precipitate the same amount of protein from muscle under similar conditions; consequently, agreement of quantitative results is not evidence of qualitative similarity unless substantiated by other proof.

The evidence of critical zones and the increment of salt are such as to suggest that we are perhaps not dealing with the extraction and precipitation of individual proteins, but with a phenomenon of the periodic precipitation of protein which reflects the activity of the salt, or to the periodic reaction between a colloid and the added electrolyte found by Holker (12). We are inclined to believe, however, that the phenomenon involves chemical characteristics of the protein as well as physical changes. The solution of the problem is not a simple one; the question of the preparation of the fractions without denaturation, which is particularly difficult with the proteins of muscle, and a ready means of identification which is not involved in the separation of the protein are related problems which must first be solved.

Evidence of the chemical individuality of protein fractions has been found in the differences in the distribution of the amino acids. There is also considerable evidence with regard to the fraction specificity of proteins when tested by immune reactions. In addition to these observations there are those which relate to the changes in the quantitative distribution of the various fractions in young animals, such as, (*a*) the definite breaks in the curve of precipitation in which no precipitation occurs, but in which there is a precipitate at the concentrations of salt which precipitate the fraction just before or just after the missing fractions. Such a condition exists in the blood plasma of the new-born calf which does not contain Fractions II and III (Table II), but does have Fractions I and IV. Upon feeding colostrum,

Fractions II and III appear in the blood plasma; but not upon feeding milk. (*b*) There are definite changes in one fraction apparently independent of the others, such as occur in the case of fibrinogen (Fraction I), or in the calf with increasing age (13). (*c*) There are characteristic differences in the quantitative distribution of the various protein fractions in different animals. (*d*) We have noted certain cases in which enormous changes occur in the quantity of Fractions I to IV without any apparent change in Fractions V to IX. (*e*) The ultramicroscopic granules of Bottazzi and Quagliariello no longer appear in muscle with concentrations of salt higher than Fraction I, or occasionally Fraction II. Such evidence is of biological origin and may possibly be interpreted in another manner upon further study; it stands as presumptive evidence of the individuality of the protein fractions and it is an impediment to the acceptance of a periodic phenomenon which is the expression of the action of the solvent on a solute of uniform composition and possibly variable structure, such as Holker describes, or of an interrelated series of colloidal particles of different degrees of dispersion as postulated by Herzfeld and Klinger (14).

Protein Fractions of Muscle —The work of Halliburton and the later critical work of von Fürth are the foundation of our present conceptions of the kinds and distribution of the proteins of muscle. Von Fürth did not agree with Halliburton in regard to the properties of paramyosinogen and myosinogen nor to the number of proteins in muscle. He also modified the nomenclature of the proteins as used by Halliburton. From the data presented in this paper on the solubility and precipitation of the proteins of muscle the cause of some of the differences of these two investigators can be explained. The characteristics of the various proteins as defined by Halliburton and von Fürth are given in the following summary.

Proteins of Muscle According to Halliburton and von Fürth.[7]

Precipitant.	Halliburton.	von Fürth.
	(Did not observe soluble myogen fibrin.)	*Soluble myogen fibrin.*
Heat.		30–40°C.
Salts.		Precipitated by the same salt concentration as myogen.
	Paramyosinogen.	*Myosin.*
Heat.	47°C.	47–50°C. (52°C. in preparations of myogen.)
$MgSO_4 + 7\ H_2O$	37–50 gm. to 100 cc. of a 5 per cent solution (1.75 mols).	
NaCl	15–26 gm. to 100 cc. of a 10 per cent solution (3.94 mols).	
$(NH_4)_2SO_4$		12–24 per cent (1.00–1.75 mols). Uses 23 per cent in preparations.
Dialysis.	Precipitated.	Precipitated.
Acids.	Not precipitated.	" by acetic acid, mineral acids, and CO_2.
Alcohol.		Precipitated — loses its solubility on standing.
	Myosinogen.	*Myogen.*
Heat.	56°C.	55–65°C.
$MgSO_4 + 7\ H_2O$	60–90 gm. to 100 cc. of a 5 per cent solution (2.64 mols).	Partial precipitation by saturated solution.
NaCl	30–36 gm. to 100 cc. of a 10 per cent solution (5.38 mols).	" "
$(NH_4)_2SO_4$		26–40 per cent (2.00–3.75 mols). Uses 29 per cent (2.25 mols) as the upper limit for precipitation in his preparations.

[7] We have used the nomenclature of each author when considering his data. Molar concentrations have been estimated by us.

Precipitant.	Halliburton.	von Fürth.
Dialysis.	Precipitated.	Not precipitated.
Acids.		" " from a salt-free solution, but is precipitated in the presence of salt.
Alcohol.		Precipitated—becomes insoluble on standing.
	Myoglobulin.	
	Resembles serum globulin in its properties. The most marked difference is in its coagulation temperature, 63°C. Coagulation temperature of serum globulin, 75°C.	Could not distinguish myoglobulin from myosin.
Heat.	63°C.	
$MgSO_4 + 7\ H_2O$	94 gm. to 100 cc. of a 5 per cent solution to a saturated solution.	
NaCl	Saturated solution.	
	Muscle Albumin.	
	Is probably identical with serum albumin *a*.	Could not identify.

Von Fürth came to the conclusion that Halliburton's myosin, the coagulation product of paramyosinogen and myosinogen, was not composed of two proteins but one, the paramyosinogen of Halliburton or the myosin of Kühne.

An explanation of the different conclusions of Halliburton and of von Fürth involves a consideration of the procedures followed in the preparation of the muscle extracts.[8] Halliburton perfused his animals with cold sodium chloride solutions and, after removing the muscle and cutting it in pieces, squeezed muscle plasma from the frozen muscle-snow with a cooled lemon squeezer or extracted the finely divided frozen muscle with salt solutions of various strengths, 5 per cent $MgSO_4$, 10 per cent NaCl, one-

[8] We shall follow Halliburton's nomenclature in the following discussion.

half saturated Na_2SO_4, and filtered the extract through filter paper. Some extracts were made at room temperature and at 37°C. Extracts were obtained similar to those produced by extraction in the cold. The amount of protein extracted was higher with the frozen muscle. Von Fürth, on the other hand, perfused his animals with salt solution warmed to 36–40°C., added 0.6 per cent NaCl solution to the hashed muscle at room temperature or in the ice chest, and finally obtained the extract by squeezing the mixture in a tincture press. The operation required about ½ hour after the death of the animal.

Stewart and Sollmann (15) employed a slightly different procedure for extraction from either Halliburton or von Fürth. They extracted with 5 per cent $MgSO_4$ for 24 to 48 hours at 20°C. with a relatively large amount of solution, 5 parts of solvent to 1 part of muscle, strained off the liquid, squeezed out the residue, and finally filtered the solution through filter paper. The variable quantitative results obtained by these men are probably to be ascribed to the long period of standing; under the best conditions their data are probably comparable to our results.

Von Fürth chose 0.6 per cent NaCl solution for rabbit muscle because he believed that higher concentrations of salt were not indifferent solvents. It is evident from our results that 0.109 molar phosphate solution, which gives extractions equal to those with 0.8 per cent NaCl solution, fails to extract or takes out only a part of the protein fraction (or fractions) which is precipitated at 1.125 molar solution. In other words, it does not extract a large proportion of the muscle protein to which Halliburton gave the name paramyosinogen, or which includes the fractions which contain Bottazzi's ultramicroscopic granules, the proteins which exhibit the most swelling in acid and are probably the most characteristic of muscle. The precipitation limits which von Fürth gave for his myosin, which he considers as equivalent to Halliburton's paramyosinogen, indicate that he was not obtaining the same effects with 0.6 per cent NaCl as did Halliburton with his solvents.

From our work on the relative solution and precipitating capacities of salts, $MgSO_4$ and $(NH_4)_2SO_4$ show the same capacity for muscle as for serum. It is evident, therefore, that the substance which von Fürth studied as myosinogen required a

much higher concentration of salt for the precipitation than did Halliburton's myosinogen—they were not the same substances. Von Fürth's paramyosinogen preparations on the basis of salting-out would consist of Halliburton's paramyosinogen plus myosinogen and his myosinogen was precipitated in the region where albumin is precipitated in blood serum and would correspond to Halliburton's myoalbumin. The choice of the original solvent is undoubtedly the basis of the difference between the findings of Halliburton and von Fürth. The quantity of the most labile protein fraction of muscle, which was included in Halliburton's paramyosinogen, obtained by von Fürth, was probably small and variable. The difference in the relative proportions of the various proteins found by the two men is also explained on the above basis of difference in the solvent.[9] It is to be remembered that while von Fürth made use of salt solutions for the preparation of his extracts the proteins were identified by their coagulation temperatures.

In interpreting our results we are inclined to consider the data of von Fürth as supplementing those of Halliburton. The following relations would then exist.

(*a*) *Paramyosinogen.*—Halliburton's paramyosinogen corresponds to the protein precipitated between the points of maximum extraction, 0.225 or 0.525 molar potassium phosphate, and 1.125 molar potassium phosphate (Fraction I, Table II). If the critical zones are to be accepted as evidence of the presence of a protein fraction there are two proteins included in paramyosinogen as defined above.

Bottazzi and Quagliariello's ultramicroscopic granules appear in this fraction of the muscle proteins. Muscle extracts at 0.225 or 0.525 molar potassium phosphate invariably show ultramicroscopic particles in the dark-field microscope and an

[9] It is probable that the cold water extracts of meat studied by Grindley and his associates (16) and by Moulton (17) relate largely to proteins which are soluble in 1.425 molar potassium phosphate, or hydrolysis products of the more labile protein fractions, and do not include the proteins soluble in lower concentrations of salt to any considerable extent. Stewart and Sollmann (15) showed that water was not so good a solvent as 5 per cent $MgSO_4$ and assumed that probably 0.6 per cent NaCl would also be a poor solvent. They assumed that this might be an explanation of some of von Fürth's results.

opalescence by reflected light. The opalescence persists to Fraction I (Table II) for the various salts. Fraction I is often clear, but is sometimes opalescent, while Fraction II is always clear. A water extract of muscle is as a rule clear without opalescence or only a trace of opalescence. We are not entirely sure that Bottazzi's interpretation that these granules exist normally in muscle is correct. Our data as a whole are in favor of such an hypothesis, but the manner in which the opalescence may increase without aggregation, *i.e.* increase in size of the particles as observed in the dark-field, suggests that they are a post-mortem change.

(*b*) *Myosinogen.*—Halliburton's myosinogen agrees in most of its properties with von Fürth's myosin. It would be included in Fractions II and III, 1.125 to 1.725 molar potassium phosphate. On the basis of our work on blood proteins and muscle proteins there would be two protein fractions included in myosinogen.

(*c*) *Myoglobulin.*—The myoglobulin of Halliburton would correspond to our Fraction IV, precipitated between 1.725 and 2.025 molar potassium phosphate. Von Fürth denies the presence of such a protein. There is no collateral evidence for such a fraction, except such as exists for blood serum. The quantity of nitrogen found in this fraction is greater than could be accounted for on the basis of the blood which might be present in the muscle.

(*d*) *Myoalbumin.*—Von Fürth's myogen corresponds in its precipitation limits to those of blood or egg albumin. The coagulation temperature does not correspond with that of the usual albumin. We have not noted any particular lability of the fraction extracted by 2.025 molar potassium phosphate. Preparations have been made of muscle protein with ammonium sulfate in the region of salt concentrations given by von Fürth which exhibited the properties of albumin as generally accepted.

Procedure for the Determination of the Distribution of the Protein Fractions of Muscle.

Extract 5 gm. portions of finely divided muscle, from which extraneous fat and connective tissue have been removed, with 50 cc. of the following concentrations, volume-molar, of a mix-

ture of monopotassium phosphate and dipotassium phosphate in the ratio of 1:2: 0.225, 0.525, 0.825, 1.125, 1.425, 1.725, and 2.025, and also with an 8 per cent solution of trichloroacetic acid. The concentrations of the phosphate mixture should be greater than the desired final concentration by the amount necessary to bring the water of the meat to the desired concentration. We have assumed 4 cc. of water in 5 gm. of meat, without reference to the salt contained in the meat. This assumption has agreed fairly satisfactorily with the water content of the muscle examined. The extraction should extend over approximately $1\frac{1}{2}$ to 2 hours. The tubes must be inverted from time to time.[10,11] Filter the extracts on fairly close, dry, filter papers. Determine the nitrogen in aliquot samples of the filtrates. Total nitrogen and moisture are determined on separate samples.

Comments.—In grinding the muscle a cutting machine is to be preferred to a grinder which forces the meat against a plate. We have obtained very good results, however, with an Enterprise grinder and using a cutting plate with holes 5/64 inch in diameter for the last grinding. For the extraction we have used large Pyrex test-tubes, 22 × 200 mm., such as are used in blood analysis, fitted with rubber stoppers. The extract is filtered into large test-tubes. The funnels are placed directly in the tubes. When the funnel is covered with a watch-glass the filtration system is reasonably protected from evaporation. It is desirable to have the solutions prepared and ready before an experiment for then the meat can be weighed out and the

[10] A shaker such as a slowly rotating wheel which does not cause a violent agitation of the contents of the tubes is desirable. In our work we have resorted to shaking by hand because of the large number of determinations which it was necessary to make on some experiments.

[11] The following weights of the two phosphates, anhydrous salt, will give the concentration indicated. To increase the concentration for 5 gm. of muscle plus 50 cc. of solution multiply by 1.08. The weights are for 1 liter of solution.

Molar concentration	0.225	0.525	0.825	1.125	1.425	1.725	2.025
KH_2PO_4	10.21	23.83	37.45	51.06	64.68	78.29	91.91
K_2HPO_4	26.14	61.00	95.84	130.68	165.53	200.38	235.24

extraction begun as soon as possible after the death of the animal. The samples to be extracted at 0.225 and 0.525 molar solution of potassium phosphate should be weighed out first.

Calculation and Interpretation of Results.

The maximum extraction of muscle protein is obtained at 0.225 to 0.525 molar solution of potassium phosphate (or the equivalent concentration of another salt). The values for the various protein fractions are obtained by subtracting the quantity of nitrogen obtained at any given concentration from that for the preceding fraction. In calculating the results obtained upon analysis in terms of 100 gm. of muscle we have assumed that the proteins are uniformly distributed throughout the solution (which includes the water of the muscle). The calculation is, therefore, based, in the case of 50 cc. of added solution and 5 gm. of muscle, on 54 cc. of extract. The water associated with the non-soluble portion of muscle is assumed to be capable of dissolving salts and soluble protein.

In interpreting the analytical results in terms of the proteins described by Halliburton and von Fürth the following calculations may be made. $(K)PO_4$ will be used to designate the mixture of phosphates.

Paramyosinogen = Nitrogen in the extract at 0.225 or 0.525 molar $(K)PO_4$ minus nitrogen in the extract at 1.125 molar $(K)PO_4$.

Myosinogen = Nitrogen in the extract at 1.125 molar $(K)PO_4$ minus nitrogen in the extract at 1.725 molar $(K)PO_4$.

Myoglobulin = Nitrogen in the extract at 1.725 molar $(K)PO_4$ minus nitrogen in the extract at 2.025 molar $(K)PO_4$.

Total globulin = Nitrogen in the extract at 0.225 or 0.525 molar $(K)PO_4$ minus nitrogen in the extract at 2.025 molar $(K)PO_4$.

Albumin = Nitrogen in the extract at 2.025 molar $(K)PO_4$ minus nitrogen in the extract with trichloroacetic acid.

Non-protein nitrogen = Nitrogen in the extract with trichloroacetic acid.

In our analyses we have chosen to include each fraction between 0.525 molar $(K)PO_4$ and 2.025 molar $(K)PO_4$ in which case the fractions are numbered according to Table II. On the other hand, in the following tables we have also grouped Fractions *A* to I as paramyosinogen and Fractions II to IV as myosinogen.

In this study no attempt has been made to differentiate the nitrogen present as hemoglobin nitrogen, nor to fractionate the non-protein nitrogen. The extracts are deeply colored through those obtained with a 2.025 molar $(K)PO_4$ solution. The hemoglobin is largely precipitated in the albumin fraction. Grindley and his associates (16) have developed procedures for the determination of the non-protein constituents in the cold water extracts of muscle. The use of trichloroacetic acid for the estimation of non-protein nitrogen has given very good results which are comparable with those obtained with a 3.75 molar solution of sodium sulfate at 37°C., which is equivalent to a 3.25 molar solution of ammonium sulfate, essentially a saturated solution of ammonium sulfate.

Analyses of the Muscle of the Calf, Cow, and Rabbit.

In the accompanying table (Table V) are presented certain data on the calf, cow, and rabbit. Only data on samples taken immediately after death are given. The calves and rabbits were killed at the laboratory. The samples were taken from the hind leg, the gracilis and semimembranosus muscles of the calf, and all the muscles in the case of the rabbit. In the case of the cows and the bull, samples were obtained at the abattoir and were taken from the exposed muscle near the symphysis pubis as the carcass is halved. Sufficient muscle for moisture determinations was not obtained.

From a consideration of the data it is evident that the most marked differences between the calf and the cow are in the quantity of Fractions *A* and I, *i.e.* the paramyosinogen of Halliburton, which are as a rule higher in the cow than in the calf. There was a marked difference in the texture and color of the different samples of muscle from the cows. The absolute amount of paramyosinogen in the rabbits was even higher than that of the cows.

When the relative proportions of paramyosinogen, myosinogen, and albumin are calculated we find (Table VI) that the cow has a higher percentage of total soluble nitrogen and of paramyosinogen nitrogen than the rabbit. The calf has a slightly higher proportion of insoluble nitrogen than either the cow or rabbit.

TABLE V.

Data Relating to the Differential Fractionation of Proteins in the Muscle of the Calf, Cow, and Rabbit. Results Are Expressed as Grams of Nitrogen in 100 Gm. of Fresh Muscle.

Age.	Moisture.	Total N.	Total soluble N.	A	I	"Paramyosinogen" N.	II	III	IV	"Myosinogen" N.	Total globulin N.	Albumin V to IX.	Non-protein N.
						Calf.							
days													
2	76.5	3.01	1.42	0.22	0.18	0.30	0.06	0.22	0.23	0.51	0.92	0.11	0.39
2	78.7	2.91	1.28	0.17	0.18	0.35	0.20	0.08	0.12	0.40	0.75	0.12	0.41
2	75.4	3.12	1.51	0.31	0.28	0.59	0.19	0.07	0.11	0.37	0.99	0.30	0.36
2*	76.8	3.09	1.44	0.25	0.31	0.56	0.14						
3	76.4	3.18	1.58	0.20	0.37	0.58	0.15	0.09	0.18	0.42	1.00	0.18	0.40
5	71.7	2.99	1.46	0.37	0.18	0.55	0.21	0.02	0.16	0.39	0.93	0.25	0.27
8	79.2	2.83	1.13	0.06	0.21	0.27	0.22	0.06	0.08	0.36	0.62	0.15	0.42
11	74.8	3.09	1.10	0.12	0.25	0.37	0.10	0.12	0.05	0.27	0.64	0.08	0.38
20	75.8	3.17	1.65	0.36	0.31	0.67	0.18	0.14	0.15	0.48	1.15	0.21	0.29
26	79.1	3.14	1.50	0.37	0.14	0.51	0.26	0.14	0.11	0.51	1.02	0.10	0.38
44	70.3	3.24	1.56	0.22	0.39	0.61	0.13	0.13	0.13	0.38	0.99	0.20	0.36
Average.....	75.8	3.07	1.42	0.24	0.25	0.48	0.17	0.11	0.13	0.41	0.90	0.17	0.37
						Adult cow and bull.							
± 8 mos.		2.81	1.90	0.59	0.21	0.80	0.21			0.50	1.30	0.18	0.41
Bull.		2.81	1.68	0.52	0.14	0.66	0.10	0.15	0.15	0.40	1.16	0.19	0.43
Cow.		2.89	1.68	0.45	0.24	0.69	0.14						0.39
"		3.04	1.35	0.36	0.18	0.54	0.10	0.06	0.15	0.31	0.85	0.11	0.39
"		3.19	1.85	0.25	0.40	0.65	0.17	0.21	0.14	0.52	1.17	0.23	0.45
Average.....		2.95	1.69	0.43	0.23	0.67	0.14			0.43	1.09	0.18	0.42
						Adult rabbit.							
	73.8	3.67	1.69	0.54	0.09	0.63	0.18	0.09	0.19	0.55	1.09	0.16	0.45
	74.6	3.55	1.85	0.56	0.14	0.70	0.16	0.20	0.19	0.55	1.25	0.17	0.43
	74.3	3.52	1.78	0.54	0.19	0.73	0.20	0.11	0.19	0.50	1.23	0.11	0.44
	73.3	3.53	1.83	0.50	0.30	0.80	0.11	0.11	0.22	0.44	1.24	0.15	0.44
Average.....	74.0	3.57	1.79	0.53	0.18	0.72	0.16	0.13	0.20	0.51	1.20	0.15	0.44

* Not included in the average.

It is difficult to correlate the data which we have presented with those of previous investigators. All previous work, with the exception of that of Moulton (17), has relied upon the temperature of coagulation for the estimation of the protein fractions, while the basis of identification has been dependent, in part, upon the salting-out procedure. It is apparent that the largest protein fractions are in the group which comprises the globulins and that there is slightly more "paramyosinogen" than there is of the remainder of the globulin fractions which we have grouped in Tables V and VI as "myosinogen." Albumin repre-

TABLE VI.

Average Proportions of the Various Fractions of Protein in the Muscle of the Calf, Cow, and Rabbit, and the Percentage Distribution of These Fractions.

	Total N.	Total soluble N.	"Paramyosinogen" N.	"Myosinogen" N.	Total globulin N.	Albumin N.	Non-protein N.	Insoluble N.
Calf.								
Nitrogen, *gm.*	3.07	1.42	0.48	0.41	0.92	0.11	0.39	1.65
Total nitrogen, *per cent.*		46.2	15.6	13.4	30.0	3.6	12.7	53.7
Cow.								
Nitrogen, *gm.*	2.95	1.69	0.67	0.43	1.01	0.18	0.42	1.53
Total nitrogen, *per cent.*		57.3	22.7	14.6	34.2	6.1	14.2	51.9
Rabbit.								
Nitrogen, *gm.*	3.57	1.79	0.72	0.51	1.20	0.15	0.44	1.78
Total nitrogen, *per cent.*		50.1	20.2	14.3	33.6	4.2	12.3	49.9

sents the smallest proportion of the soluble muscle proteins. The insoluble protein nitrogen is approximately one-half the total nitrogen of muscle. This is much higher than the 8 per cent found by Saxl (18) for striated muscle, but agrees fairly well with his results for heart muscle.

If we accept our interpretation of the proteins extracted by water as representing chiefly those extracted at 1.425 molar potassium phosphate, then the results for globulin and albumin obtained by Moulton (17) are in fair agreement with the data presented above.

The changes which take place in muscle, exclusive of the autolysis which occurs, upon standing in the refrigerator relate particularly to the fraction between 0.525 and 1.125 molar potassium phosphate. There is very little change in the quantity of protein extracted at higher concentrations of potassium phosphate. There is a gradual decrease in the water-soluble nitrogen and an increase in the non-protein nitrogen. Data illustrating these changes in rabbit muscle are contained in Table VII. Similar data have been obtained for the muscle of the calf.

TABLE VII.

Data Showing the Changes Which Take Place in the Proteins of Muscle of the Rabbit when Stored in the Refrigerator. Results Are Expressed as Grams of Nitrogen Extracted per 100 Gm. of Muscle.

Concentration of $(K)PO_4$.	H_2O	0.225	0.525	0.825	1.125	1.425	1.725	2.025	Non-protein N.
Immediately.....	0.92	1.85	1.85	1.54	1.05	0.99	0.79	0.60	0.43
After 2 days.....	0.91	1.38	1.35	1.35	1.05	0.99			0.44
" 4 "	0.85	1.38	1.37	1.34	1.09	1.04			0.50
" 9 "	0.83								0.50

SUMMARY.

1. A procedure has been developed for the differential estimation of the proteins of muscle which is designed primarily to obtain results on the muscle before certain of the proteins have undergone a postmortem change in solubility.

2. A relation has been shown to exist between the concentrations of salt required for the extraction of muscle and those required for the precipitation of the proteins of blood. On the alkaline side of the isoelectric point of the muscle proteins for any anion the cation is the determining factor in extraction and precipitation.

3. The observations of Halliburton and of von Fürth on muscle proteins have been, in part at least, correlated.

4. Data on the distribution of various protein fractions in the muscle of the calf, cow, and rabbit and in the muscle of the rabbit following a period of storage, have been presented.

We wish to thank Miss Olivia Duffy for her assistance in the technical work in these experiments.

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THE DETERMINATION OF GASES IN BLOOD AND OTHER SOLUTIONS BY VACUUM EXTRACTION AND MANOMETRIC MEASUREMENT. I.*

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CONTENTS.

		PAGE
I.	Principles of construction and use of the manometric apparatus	523
II.	Details of apparatus	527
III.	Details of general technique	531
IV.	Calculation	538
V.	Determination of carbon dioxide in blood or plasma	543
VI.	Determination of plasma carbon dioxide capacity	551
VII.	Determination of oxygen in blood	554
VIII.	Combined determination of oxygen and carbon dioxide in blood	561
IX.	Determination of carbon monoxide in blood	562
X.	Combined determination of carbon dioxide, oxygen, and carbon monoxide	564
XI.	Micro analyses	565
XII.	Determination of gases in liquids saturated at high tensions	568
XIII.	Determination of dissolved gases in water	571
XIV.	Use of the apparatus for air and general gas analysis	572

I. Principles of Construction and Use of the Manometric Apparatus.

The method reported in the present paper resembles that previously described by Van Slyke (1917) and by Van Slyke and Stadie (1921) in that the gases are extracted from solution by shaking the latter in a relatively large free space in a Torricellian vacuum, and in that for subsequent measurement the volume of the extracted gas is reduced and the pressure increased. In the former

*A preliminary note on the apparatus described in this paper was published in the Proceedings of the National Academy of Science (Van Slyke, D. D., *Proc. Nat. Acad. Sc.*, 1921, vii, 229).

"volumetric" method, however, the pressure was brought always to atmospheric, and the gas volume was read on a scale; while in the present "manometric" method the volume is brought to an arbitrarily chosen size, and the amount of gas is determined from the pressure exerted on a manometer.

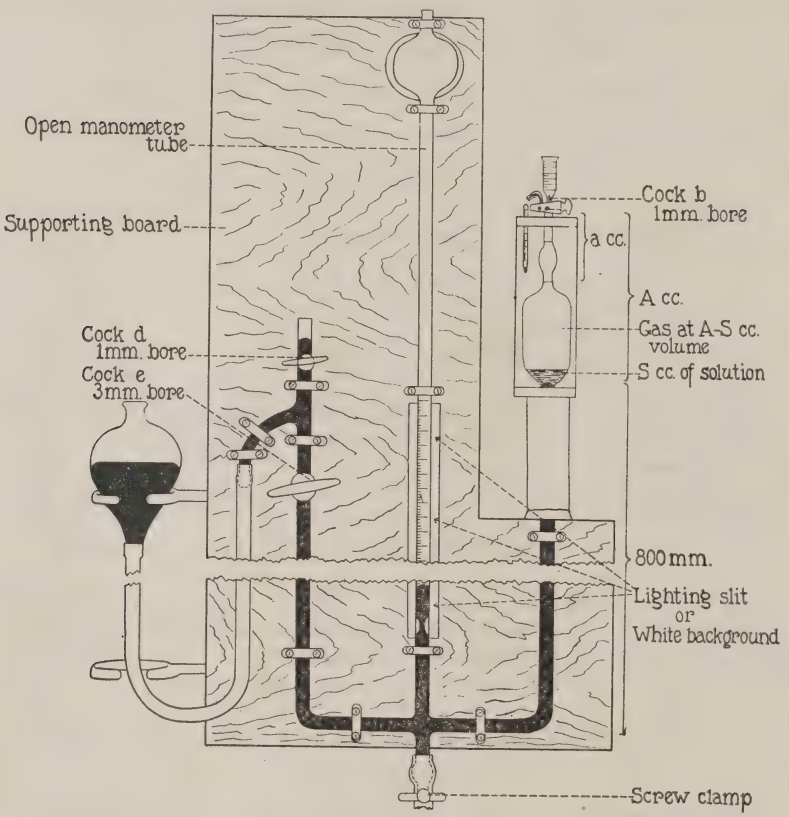

FIG. 1. Apparatus, with solution in position for extraction of gases. Manometer is of open type.

Under the conditions of the former method, when all measurements were made at atmospheric pressure, the error in volume reading might be 10 or even 100 times that of the pressure measurement. The advantage of the present method is that it permits the analyst to choose the magnitude of both the volume and the pressure which he measures. He may accordingly fix them within such limits that the errors in measuring both are of the same order

of magnitude. Thereby the percentage error in gas measurement becomes the minimum attainable with given absolute errors in pressure and volume.

The apparatus consists of a short pipette with the upper stem closed by a stop-cock, the lower connected with a glass tube. The

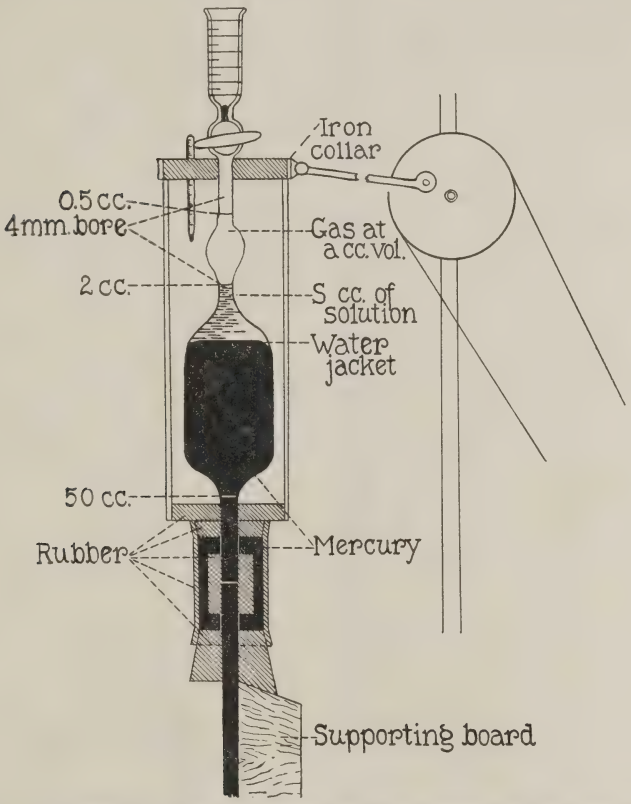

Fig. 2. Extracting chamber, showing mercury-sealed rubber joint at bottom and attachment of Stadie shaker at top. Gas is at 2 cc. volume for pressure reading.

latter descends 800 mm., then turns at a right angle to connect with a leveling bulb and a mercury manometer, which may be either open at the upper end (Fig. 1) or closed (Fig. 3). The pipette is calibrated at two points to hold a cc. of gas for pressure measurement and A cc. of total volume, respectively, as shown in Figs. 1 and 2.

For analysis the sample of blood or other solution is introduced into the chamber over mercury, together with the reagents to free the desired gases from combination. A Torricellian vacuum is

Fig. 3. Apparatus with closed manometer.

obtained, as in the previous "volumetric" apparatus, by lowering the leveling bulb, and the gases are extracted from solution by 2 or 3 minutes shaking. The gas volume is then reduced

to a cc. by admission of mercury through cock e, and the reading p_1 is made on the manometer. The gases are either ejected or are absorbed by proper reagents, and the reading p_2 is taken, with the same gas volume. The partial pressure P of the gas at a cc. volume is then $P = p_1 - p_2$ mm. of mercury, from which the gas volume at 0°, 760 mm. may be calculated. The calculation is simpler than with the constant pressure apparatus, because the barometric pressure is not a factor. No corrections are required for vapor tension or for capillary attraction in the manometer tube, since these factors are the same at both readings. It is assumed that the temperature in the apparatus is the same when the p_1 and p_2 readings are taken. The time interval between the two readings is so brief (1 to 4 minutes) that sufficient constancy is attained (within 0.1°C.) by the insulation afforded by the water jacket. When the open manometer is used, constancy of barometric pressure over the same time interval is also assumed.

The total volume A of the chamber is a matter of convenience, but it is desirable to have it so large that the greater part of the dissolved gases shall be extracted. For analysis of 1 cc. of blood convenient magnitudes are $A = 50$, $a = 2$, $S = 3.5$ to 7.0 cc. (see Fig. 1). At 20° 0.01 cc. of gas under these conditions gives a reading of $P = 3.9$ mm. For minute amounts of gas an additional a mark at 0.5 cc. is desirable. At this volume 0.01 cc. of gas exerts about 16 mm. pressure.

For the most precise CO_2 determinations we have employed an analysis chamber in which all the dimensions are twice those given above, and in which as much as 3 cc. of blood could be analyzed for CO_2 with an error not exceeding 1 part in 500. For micro analyses of 0.2 cc. blood samples we have used both the 50 cc. chamber and a 10 cc. chamber, with results approximating 1 per cent accuracy. The different chambers can all be used on the same manometer.

II. Details of Apparatus.

The *extraction chamber* is simpler than that of the former "volumetric" apparatus; it is calibrated at only three points (*e.g.*, 0.5, 2, and 50 cc.), and has no cock at the bottom. The

lack of necessity for a long narrow graduated tube for measurement of varying volumes of gas makes the chamber short and convenient to shake. The short length of space *a* also simplifies the problem of drainage.

The *mercury seal*, shown in Fig. 2, around the rubber joint at the bottom of the chamber was found necessary. Even thick pressure tubing, exposed to the air, permits the diffusion, during extraction *in vacuo*, of sufficient amounts of air to affect pressure readings as accurate as those that can be made on the present apparatus.

For details of the *mechanical shaker*, see Stadie (1921). The driving shaft should not exceed 8 or 9 cm. in length. Too long a shaft causes wobbling of the chamber sideways during the shaking.

Stop-cock *d* offers a convenient means of expelling the air which gradually diffuses through the rubber tubing connected with the leveling bulb and collects below this cock.

The screw clamp at the bottom of the manometer permits withdrawal of the mercury.

The *open manometer tube* is of 4 mm. inner diameter, and is expanded into a bulb at the top to prevent loss of mercury when the leveling bulb is raised high to expel solutions from the analyzing chamber. At the bottom it is contracted as shown in Fig. 1, to 1.5 mm. bore to minimize the tendency of the mercury to oscillate between the manometer and the analysis chamber when cock *e* is closed.

Alternative Closed Manometer Tube.—Instead of ending at the top with an open bulb as in Fig. 1, the manometer tube may be closed with a cock at the top, as shown in Fig. 3. The air is expelled before the manometer is used, so that there is no atmospheric pressure on the mercury surface in the tube. Consequently all the readings are about 800 mm. higher from the laboratory floor than in the open manometer. The closed manometer is of advantage from the standpoint of comfort, inasmuch as the operator does not need to bend down to take the zero readings.

The closed manometer tube is contracted to 1 to 1.5 mm. a little below the cock at the top, as shown in Fig. 3, to lessen the force with which the rising mercury strikes the cock.

Slight amounts of moisture find their way into the closed manometer as the mercury flows back and forth from the analysis chamber. Some error from the vapor pressure of this moisture would be probable, as the manometer tube is not protected against sudden temperature changes. To absorb the water vapor, 2 or 3 drops of concentrated sulfuric acid are admitted through the cock at the top and permitted to flow down the tube for about 10 cm. Mercury is then forced up through the cock, leaving behind enough sulfuric acid to wet the upper end of the manometer, but not enough to flow down and interfere with readings of the mercury meniscus. The acid is renewed occasionally in this manner.

The closed manometer has the theoretical advantage over the open manometer in that the former is not affected by change in barometric pressure during the time interval between the p_1 and p_2 readings. In the 1 to 4 minute intervals, however, this is a negligible factor. The closed manometer is obviously not quite so simple as the open one. The latter has been used for most of the analyses thus far performed, but in comparisons of the two, identical results have been uniformly obtained.

For *the manometer scale* we at first stretched a steel meter tape divided into millimeters behind the tube, or sunk a brass scale into the board. The most satisfactory readings, however, have been made with the scale, about 600 mm. long, etched on the manometer tube itself. The marks are 1 millimeter apart, and are in the form of semicircles, passing around the tube from the middle of the front to the back. The centimeter and half-centimeter marks are complete rings. By sighting the meniscus across these marks error from parallax is avoided.

A good background is provided by a strip of white paper or of mirror glass behind the manometer. A still more satisfactory background is given by an illuminated strip of frosted glass, as shown in Fig. 1. From the board behind the manometer a section about 12 mm. wide is cut out. The rear of the aperture is covered by the strip of frosted glass, which is illuminated from behind by two electric lights, each in a tubular bulb of frosted glass 30 cm. long and 3 cm. wide. In order to avoid heating the manometer, the lights are turned on only at the moment a reading is made. The switch for them is conveniently attached to the same board with the apparatus. A practised observer, with the

help of a reading glass, can approximate an accuracy of 0.1 mm. in his readings, either with the etched manometer tube or with a scale behind the tube, but the attainment of such accuracy is considerably easier with the etched tube.

It is advisable, before each reading, to tap the manometer tube with the finger. There may be a slight lag on the part of the mercury in adjusting itself to final pressure conditions, and a consequent error in the reading unless this precaution is taken.

Range of the Apparatus.—As stated before, for maximum accuracy the analyst chooses such sizes of *a* and of the blood or other sample analyzed, that the percentage errors in measuring the gas volume of *a* cc. and the resultant pressure of *P* millimeters are about equal. By so doing it is possible with the larger apparatus to approach a constancy of 2 *pro mille*. The precision of the manometer readings, however, enables one to determine a wide range of gas magnitudes with accuracy sufficient for most purposes (*e.g.* 1 per cent), in a single apparatus. In the 50 cc. apparatus, with *a* volumes of 0.5 and 2.0 cc., we have analyzed satisfactorily liquids containing from 0.2 to over 100 volumes per cent of gas (O_2 or CO_2), and have used blood samples varying from 0.2 to 2.0 cc.

Calibration of Apparatus.—The volume from the *bottom* of cock *b* down to the *a* mark is determined by weighing the water delivered by the technique described in the paper[1] by Van Slyke and Stadie (1921). Since in the present apparatus only two *a* volumes are used, calibration is simpler than in the case of the former "volumetric" apparatus with its graduated scale, and correction curves are unnecessary.

The volume between cock *b* and *A* mark is similarly determined. When *A* is 50 cc., an accuracy of 0.2 cc. is sufficient.

The cup at the top of the chamber is graduated in 0.5 cc. divisions *with the zero mark at the point where the bottom of the cup joins the capillary.* When solutions are measured into the cup, the capillary is filled with mercury.

Simplified Construction of Apparatus.—The apparatus may be constructed in any laboratory with details slightly less convenient, but not essentially less accurate than those above outlined. The manometer tube and the two tubes connecting with the manom-

[1] Van Slyke and Stadie (1921), p. 4.

eter on the right and left, instead of being fused together, may be joined to a central + tube with very heavy pressure tubing. The rubber joints are reinforced against stretching under mercury pressure by wrapping them firmly with adhesive tape. Instead of the millimeter scale etched on the glass, a metal meter stick or metallic tape may be fastened to the board behind the manometer tube. The analysis chamber may be made from a sturdy 50 cc. pipette by fusing a cock on the stem. The cock, although conveniently 3-way, as in Fig. 1, may be 2-way, particularly if suction is available for drawing off used solutions. The Stadie shaking device needs to be well constructed, and requires the services of a mechanic. The values of A and a in a laboratory-made apparatus will probably be such that Tables II and III for calculation will not be applicable. A table for each apparatus will need to be calculated by Equations 6 and 7 with the values for $\frac{1}{1+0.00384t}$ and α' from Table I.

III. Details of General Technique.

Measuring Samples.—Of the details essential to the precision attainable with this apparatus, none is more important than accuracy in sampling and in the introduction of samples into the analysis chamber.

In blood gas analyses, the sample must be drawn and transferred to the apparatus without exposure to air sufficient to change the gaseous composition. If the blood is not drawn directly from the vein into the pipette from which it is measured into the analyzing apparatus, it is drawn into a tube over mercury as described by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (1922). The sample is driven thence into the measuring pipette by pressure, and can be delivered from the pipette into the chamber in such a manner that exposure to the air is reduced to an ordinarily negligible minimum. The sample is admitted at a rate sufficiently slow to allow clear drainage of the pipette walls.

For measuring the samples we have adopted 0.2, 1, 2, and 3 cc. Ostwald pipettes with heavy walled capillary stems which are calibrated to deliver between marks, preferring the pipette of which neither mark is located more than 30 mm. from the bulb. It is desirable to have the 1, 2, and 3 cc. pipettes equipped with stop-cocks as in Fig. 4.

The pipette is provided with a rubber tip, made by cutting a ring about 1 cm. wide from a rubber tube with a bore of 1 or 2 mm. and walls 3 or 4 mm. thick. This tip was introduced into the technique by Poulton (1919). The ring is tapered at one end by grinding down the outer edge with sandpaper or an emery wheel,

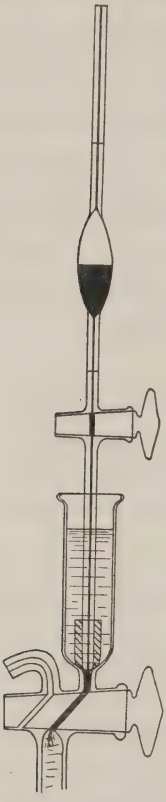

FIG. 4. Delivery of blood sample from pipette into extraction chamber of apparatus.

so that it will fit into the bottom of the cup of the gas analysis apparatus as shown inFig. 4. With the arrangement there shown, the blood sample is delivered directly from the pipette into the chamber of the apparatus. A fraction of a cc. of washing solution suffices to rinse through the residue remaining in the capillary at the bottom of the cup after the pipette has been withdrawn.

The rate of delivery of the sample by the above technique may be controlled either by the cock of the pipette, or by cock *b* or *e* of the apparatus (Fig. 1). The smoothest delivery is usually obtained by using cock *e*, the mercury surface in the open manometer at the start being adjusted at about 2 cm. above that in the chamber. With the closed manometer such adjustment is unnecessary.

With 0.2 cc. samples, pipettes with cocks and rubber tips have not been used. It is sufficiently easy to deliver so small a sample directly into the capillary at the bottom of the cup, a slow stream of reagent solution from the cup washing the sample through as it is delivered.

Adjustment of Gas Volume.—The precision of the method rests on exactness in bringing the gas to volume *a* before the pressure is measured. The calibration mark of *a* should be blackened to make it readily visible through the water jacket. It is an advantage to locate the apparatus so that light falls on it from behind the observer. A white background for the reading is secured by attaching a strip of adhesive tape 7 or 8 cm. wide to the back of the water jacket, or by locating the apparatus with a white wall behind it. Light reflected back from the white surface across the meniscus facilitates a sharp reading.

In CO_2 analyses, if in reducing the extracted gas to *a* volume the analyst fails to close cock *e* so that the fluid meniscus stops exactly at the *a* mark, readjustment must not be attempted, as moving the fluid up and down in the chamber will result in reabsorption of more CO_2 than is allowed for by the *i* correction (Equation 2). It is necessary to lower the mercury again to the *A* mark and equilibrate again for a minute.

However, after a little experience has been gained such repetition is practically never necessary. The analyst, in reducing the gas volume after extraction, opens cock *e* with a smooth gradual motion, permits the mercury to rise at a fairly rapid rate through the middle three-fourths of the chamber, and then *gradually* closes the cock and reduces the rate of flow, making the latter so slow by the time the *a* mark is reached that there is no difficulty in stopping at it exactly.

In bringing the gas to volume *a* when only gases with relatively low solubilities, such as O_2, N_2, H_2, and CO are present, reabsorp-

tion is so nearly negligible that readjustment of the *a* volume may be carried out without repeating the equilibration.

Cleaning the Apparatus between Analyses.—After both CO_2 and O_2 determinations we have found the following to be a satisfactory and very quick method of cleaning the apparatus. The residual solution from the last analysis is ejected either through the outlet tube of cock *b* or up into the cup, whence it is drawn off by suction. The level of the mercury is dropped to *A* (Fig. 1). About 1 cc. of 1 N lactic acid and 10 or 15 cc. of water are poured into the cup and run down into the evacuated chamber. The latter is then shaken for 15 or 20 seconds, and the extracted gases and solution are ejected. Such portions of the solution as remain adherent to the walls of the chamber are so nearly gas-free that they cause no error in the subsequent analysis. This rinsing must precede each analysis.

It is well occasionally to leave the chamber overnight full of chromic-sulfuric acid cleaning mixture. This is poured in through cock *b*, the fall of the mercury in the chamber being regulated by cock *e*. When the chamber is filled, *e* is closed. Cock *b* is left open, so that any gases which form may escape without forcing the cleaning mixture down into contact with the rubber connections at the bottom of the chamber.

Gas-Free Reagents.—The reagent solutions introduced before the sample (acid to free CO_2, ferricyanide to free O_2) may be freed from dissolved gases by extracting them in the apparatus before the sample is added.

The reagents added later, however, such as NaOH solutions to absorb CO_2 or hydrosulfite to absorb O_2, are made and kept air-free in properly protected reservoirs.

Small amounts (up to 30 cc.) may be prepared by extracting the solution two or three times in the chamber of the apparatus with a free vacuum space of not necessarily over 20 cc. The gas obtained after 2 or 3 minutes shaking is ejected and the extraction is repeated once or twice until no more gas is obtained. The solution is then transferred to a reservoir made from a calcium chloride tube of 50 cc. capacity, shown in Fig. 5. The glass tip is provided with a ring cut from a piece of thick walled capillary tubing. This ring is pressed against the bottom of the cup of the gas analysis apparatus, making a tight joint, through which the gas-freed

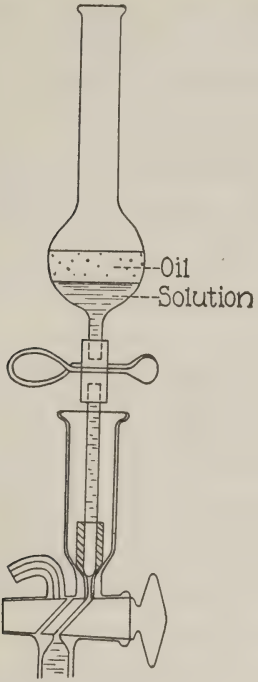

FIG. 5. Transfer of gas-free NaOH or $Na_2S_2O_4$ solution from extracting chamber to reservoir made from calcium chloride tube.

FIG. 6. Bulb for preparing and storing stable gas-free solutions.

solution is forced up into the calcium chloride tube, as shown in the figure. The layer of oil is sufficient protection against air absorption for 1 working day. The rubber tube connecting the glass tip with the reservoir should be thick walled and just long enough between the two glass tubes to permit closure by the pinch-cock. A seepage of air through the rubber into the solution occurs, and would become appreciable if the rubber connection were a long one, or thin walled.

The above method of preparation is the one we habitually use for sodium hydrosulfite, since it is desirable to make up only enough for the day's use.

For larger amounts of air-free solution, the apparatus shown in Fig. 6 is used. We employ it for preparing and storing air-free NaOH solution. It consists merely of two mercury leveling bulbs connected by a rubber tube about 1 meter long. One of the bulbs (the one shown in the figure) is fused at the top to a glass capillary. Enough solution is drawn into this bulb to fill it somewhat more than half. The cock is then closed and a Torricellian vacuum obtained by lowering the other bulb. The evacuated bulb with the solution is then shaken for 2 or 3 minutes, the pressure is raised to more than atmospheric, and the bubble of air which has been extracted is ejected. The extraction is repeated once or twice more, until no more air can be extracted.

Sodium hydrosulfite solution is made as follows: 10 gm. of powdered $Na_2S_2O_4$ are placed in a beaker. The amount is measured with sufficient accuracy by volume in a test-tube which has been marked to contain it. 50 cc. of 0.5 N KOH solution are poured over the hydrosulfite, which is immediately stirred for a few seconds with a rod. To remove some insoluble impurity that is usually present, the solution is quickly filtered through cotton. It is at once transferred to the chamber of the gas analysis apparatus, where it is freed from air and whence it is transferred to a container under oil, as described above (Fig. 5). The processes of dissolving, filtering, and transferring to the gas apparatus should be carried out as quickly as possible in order to avoid oxidation of the solution by the air.

Pyrogallol, which may be used as an alternative to hydrosulfite for absorbing oxygen, is made up in approximately saturated KOH solution (10 gm. of pyrogallic acid in 200 cc. of a solution

made by dissolving 160 gm. of KOH in 130 cc. of water). Since in this solution gases have practically zero solubility, it does not have to be made gas-free by extraction. The solution is stored under paraffin oil to prevent oxidation, and when used is delivered into the cup of the apparatus from a calcium chloride tube provided with a pinch-cock (as shown in Fig. 5).

Testing for Leaks.—Before starting a series of determinations, especially of oxygen, it is advisable to test the apparatus for leaks. A volume of water, equal to *S*, is shaken in the apparatus 2 minutes to extract the dissolved air, and the pressure is read with the gas at *a* volume. The shaking and reading are repeated. If the temperature, read on the thermometer in the water jacket, remains constant so that vapor pressure is not altered, the second and first reading should agree exactly. A leak reveals itself by a steady increase in the reading. Unless the leak is slow, the increase can be observed on the manometer while the reading is being taken.

The cause of a leak is usually improper grinding or lubrication of cock *b* (Fig. 1).

Lubrication.—There are only two cocks, *b* and *e*, to manipulate during the analyses, but it is important to lubricate both so that they turn smoothly and do not leak. We use a solution of pure para rubber gum (unvulcanized) in vaseline, 1 part by weight of the gum being dissolved with heat in 5 parts of vaseline. A thin layer of vaseline is first uniformly applied to the cock, and the latter is fitted and turned several times. The rubber lubricant is then applied in the same manner. In warm weather relatively little vaseline is used, in cold weather more is needed. The two lubricants used in this manner have proved more satisfactory than a single lubricant made by dissolving the rubber gum with larger amounts of vaseline.

Determination of Correction, c, for Manometer Depression Caused by Introduction of Absorbent Solution.—The introduction of an absorbent solution causes a certain lowering of the mercury meniscus in the chamber and hence in the manometer, merely by increasing the volume of fluid between the mercury and the *a* mark at the moment of reading (Fig. 2). This effect necessitates for the p_2 reading a correction which is determined by means of blank analyses.

The shape of the apparatus causes the area of the meniscus of the mercury in the analysis chamber to vary according to whether S is 2.0 , 3.5, or 7 cc., and a is 0.5 or 2.0 cc., such variations causing the mercury meniscus to be located at points of different cross-section in the conical upper portion of the chamber. The value of c for 1 cc. of added solution in the 50 cc. apparatus may accordingly be from 1 to 4 mm., depending on the shape of the chamber and the volume of S.

Dilute absorbents, such as 1 N NaOH, have little effect on the vapor pressure of the solution in the chamber. They give a positive value to c merely by depressing the mercury level in the chamber through increasing the volume of solution over it. A concentrated solution, such as the saturated KOH in which the pyrogallol is dissolved, has a more complex effect. It lowers the vapor tension by absorbing the water vapor in the part of the chamber above the solution. Consequently it is necessary after absorbing oxygen with pyrogallol to let the dilute solution in the chamber rise as close to cock b as possible in order to wash the concentrated alkali from the walls. Hydrosulfite as used in 20 per cent solution has no significant effect on the vapor tension. By determining c under the same conditions used in analyses vapor tension and heat of dilution effects are automatically included in the correction, but it is preferable to use absorbents without such effects.

When the final manometer reading is obtained after expulsion of the gases instead of after addition of an absorbing solution, c, of course, is zero.

IV. Calculation.

The general equation for calculating the total gas content of a solution from the amount of gas extracted in an evacuated chamber of definite volume has been developed by Van Slyke and Stadie.[2] It is expressed in their Equation 8, *viz.*

$$V_{0°,760} = V_t \frac{B - W}{760\ (1 + 0.00367\ t)} \left(1 + \frac{S}{A - S}\alpha'\right)$$

where $V_{0°,760}$ = volume of gas, measured at 0°, 760 mm., in the solution analyzed; $B - W$ = barometric pressure corrected for

[2] Van Slyke and Stadie (1921), pp. 30 and 31.

W, the vapor tension of water; t = temperature in °C.; α' is the Ostwald distribution coefficient of the gas between gas and liquid phases. $\alpha' = \frac{T}{273} \times \alpha$; where α is the Bunsen solubility coefficient. A and S have the same significance (volumes of extraction chamber and of solution in it) indicated in Fig. 1. V_t = volume of gas read at $t°$.

The equation of Van Slyke and Stadie may be applied to the present manometric apparatus by substituting a for V_t, and the partial pressure P for $B - W$ in their equation. The latter thereby becomes

$$(1) \qquad V_{0°,760} = P \times \frac{a}{760\,(1 + 0.00367\,t)} \left(1 + \frac{S}{A - S}\,\alpha'\right)$$

The solubility of CO_2 makes an empirical correction necessary, as shown by Van Slyke and Stadie[3] for the proportion of extracted CO_2 which is reabsorbed after extraction while the volume is being reduced from $A - S$ cc. to the volume at which the gas is measured; *viz.*, a cc. in the present case. Since, with $a = 2$ cc., the volume is not reduced to so low a point in the present apparatus as in that used by Van Slyke and Stadie, reabsorption is slightly less; *viz.*, about 1.4 per cent of the total CO_2 present in the system as compared with their average of 1.7 per cent. The figure may vary slightly for different apparatus, but for the same apparatus it is surprisingly constant and independent within ordinary limits of the amount of CO_2 in the sample analyzed. The determination of this factor by analysis of standard carbonates will be discussed later. Indicating as i the factor by which $V_{0°,760}$, as calculated for CO_2 by Equation 1, must be multiplied in order to correct for reabsorption, we have

$$(2) \qquad V_{0°,760} = P \times \frac{i\,a}{760\,(1 + 0.00367\,t)} \left(1 + \frac{S}{A - S}\,\alpha'\right)$$

The value of i is about 1.014 for CO_2. It is practically 1.000 for the less soluble gases, such as O_2, N_2, H_2, and CO; that is, reabsorption is negligible in their cases.

The vapor pressure (W in Van Slyke and Stadie's equation) is eliminated as a factor here, since it is the same for the reading of both p_1 and p_2 and cancels out in determining P as $P = p_1 - p_2$.

[3] Van Slyke and Stadie (1921), pp. 25 to 29.

However, the temperature effect on the specific gravity of mercury must be included. The temperature correction for reading a barometric column on a glass scale is, according to Landolt-Börnstein's "Tabellen," 0.000172 t mm. for each millimeter in the height of the barometric column. We must therefore multiply the observed mm. of partial pressure by $(1 - 0.000172\ t)$ in order to correct the results to standard conditions.

Introducing this correction, Equation 2 becomes

$$(3) \qquad V_{0°,760} = P \times \frac{i\ a\ (1 - 0.000172\ t)}{760\ (1 + 0.00367\ t)} \left(1 + \frac{S}{A - S}\alpha'\right)$$

We may simplify Equation 3 by dividing both numerator and denominator by $1 - 0.000172\ t$. Discarding negligible decimals in the resulting coefficient of t, it becomes

$$(4) \qquad V_{0°,760} = P \times \frac{i\ a}{760\ (1 + 0.00384\ t)} \left(1 + \frac{S}{A - S}\alpha'\right)$$

If it is desired to express the results in millimols instead of cc., the factor

$$\frac{i\ a}{760\ (1 + 0.00384\ t)} \left(1 + \frac{S}{A - S}\alpha'\right)$$

is divided by 22.4, the volume in cc. reduced to 0°, 760 mm. of 1 mM of any gas which acts as an ideal gas at room temperature. Such are N_2, H_2, O_2, and CO. According to Lord Rayleigh and to Le Duc (cited by Landolt-Börnstein) CO_2 also is included, although according to Guye and Pintza (cited by Landolt-Börnstein) the figure for CO_2 is 22.26 instead of 22.40. In our calculations we have used the constant 22.4 for CO_2 as well as for the other gases. If it is not exact, the correction for it in the millimolar factor is included in i, when the latter is determined as described in this paper.

Dividing the right-hand side of Equation 4 by 22.4, we have

$$(5) \qquad \text{mM gas} = P \times \frac{i\ a}{17{,}024\ (1 + 0.00384\ t)} \left(1 + \frac{S}{A - S}\alpha'\right)$$

In order to calculate volumes per cent of gas, the value $V_{0°,760}$ is multiplied by $\frac{100}{\text{cc. sample}}$, while to calculate millimols per liter the value "millimols gas" is multiplied by $\frac{1{,}000}{\text{cc. sample}}$. The total factors, therefore, by which the pressure reading is multiplied in order to give directly the concentration of dissolved gas are expressed by values in brackets in Equations 6 and 7.

(6) Vol. per cent gas

$$= P \times \left[\frac{100}{(\text{cc. sample})} \times \frac{i\,a}{760\,(1 + 0.00384\,t)} \left(1 + \frac{S}{A - S} \alpha' \right) \right]$$

$$= P \times \left[\frac{0.1316\,i\,a}{(\text{cc. sample})} \times \frac{1}{1 + 0.00384\,t} \left(1 + \frac{S}{A - S} \alpha' \right) \right]$$

$$= P \times \text{vol. per cent factor.}$$

(7) mM gas per liter

$$= P \times \left[\frac{1{,}000}{(\text{cc. sample})} \times \frac{i\,a}{17{,}024\,(1 + 0.00384\,t)} \left(1 + \frac{S}{A - S} \alpha' \right) \right]$$

$$= P \times \left[\frac{0.0587\,i\,a}{(\text{cc. sample})} \times \frac{1}{1 + 0.00384\,t} \times \left(1 + \frac{S}{A - S} \alpha' \right) \right]$$

$$= P \times \text{mM per liter factor.}$$

The values of the volume per cent factor and the millimol per liter factor for the conditions ordinarily employed are given in Tables II and III.

The factors in the table are valid for determinations in apparatus of other sizes, so long as the proportions of *A: S: a: sample* are maintained. *E.g.*, the factors for determinations on 1 cc. samples in the 50 cc. apparatus also serve for 2 cc. samples in the 100 cc. apparatus, if values of A, a, and S are likewise doubled.

In case an apparatus is used with values of i or a differing significantly from those in Tables II and III, the factors for such apparatus may be calculated from Equation 6 or 7, with the $\frac{1}{1 + 0.00384\,t}$ and α' values from Table I. For a given apparatus and type of analysis the factor $\frac{0.1316\,i\,a}{(\text{cc. sample})}$ or $\frac{0.0587\,i\,a}{(\text{cc. sample})}$ is a

TABLE I.

Data for Calculation of Factors.

Temperature.	$\frac{1}{1+0.00384t}$	α'_{CO_2}	α'_{O_2}	α'_{CO}	α'_{N_2}
°C.					
15	0.9455	1.075	0.0365	0.0268	0.0177
16	21	43	59	64	75
17	0.9387	15	53	60	72
18	53	0.989	48	56	70
19	20	66	43	53	68
20	0.9286	42	37	49	65
21	55	19	32	46	64
22	21	0.896	26	43	62
23	0.9188	73	22	40	59
24	56	50	17	36	58
25	24	28	13	34	56
26	0.9092	08	09	31	54
27	60	0.789	05	28	53
28	29	72	00	26	51
29	0.8998	55	0.0295	24	50
30	67	38	90	22	49
31	36	24	87	19	47
32	06	10	83	16	45
33	0.8875	0.696	79	13	44
34	45	82	75	10	42

The α' values are obtained by multiplying the α values by $1 + 0.00367\ t$. The α values are from Landolt-Börnstein's tables. The figures taken for CO_2 being those of Bohr and Bock, for O_2 the mean of those of Bohr and Bock and of Winkler, and for CO and N_2 those of Winkler.

$$\text{Vol. per cent factor} = \frac{0.1316\ ia}{(\text{cc. sample})} \times \frac{1}{1+0.00384\,t} \times \left(1 + \frac{S}{A-S}\alpha'\right)$$

$$\text{mM per liter factor} = \frac{0.00587\ ia}{(\text{cc. sample})} \times \frac{1}{1+0.00384\,t} \times \left(1 + \frac{S}{A-S}\alpha'\right)$$

constant, also the factor $\frac{S}{A-S}$. These two constants being determined, the rest of the calculation for the given apparatus simplifies to the form:

$$\text{Factor} = \text{constant} \times \frac{1}{1+0.0384\,t} \times (1 + \text{constant} \times \alpha')$$

TABLE II.

*Factors for Calculation of Volumes Per Cent of Blood Gases from Pressures in 50 Cc. Apparatus.**

Temperature.	Factors for CO_2.			Factors for O_2, CO, and N_2.				
	Sample =0.2 cc. S=2.0 " a=0.5 " i=1.03	Sample =1 cc. S=3.5 " a=2.0 " i=1.014	Sample =2 cc. S=7.0 " a=2.0 " i=1.014	Sample =0.2 cc. S=2.0 " a=0.5 " i=1.00	Sample=1 cc. S=3.5 "		Sample=2 cc. S=7 "	
					a=0.5 cc. i=1.00	a=2.0 cc. i=1.00	a=0.5 cc. i=1.00	a=2.0 cc. i=1.00
°C.								
15	0.335	0.2725	0.1483	0.312	0.0623	0.2493	0.0317	0.1251
16	33	11	70	10	21	85	15	46
17	31	0.2697	59	09	19	78	14	42
18	30	83	49	08	17	68	12	37
19	28	69	39	07	15	59	11	32
20	27	55	29	07	13	50	09	28
21	26	40	19	06	10	41	08	24
22	24	26	10	05	08	32	06	19
23	23	13	01	03	06	23	05	15
24	22	00	0.1391	02	04	14	03	10
25	20	0.2588	82	01	02	06	02	06
26	18	75	73	00	00	0.2398	01	02
27	17	62	64	0.299	0.0598	90	0.0299	0.1198
28	16	49	56	98	96	82	98	93
29	14	37	49	97	93	74	96	89
30	13	26	41	96	92	66	95	85
31	12	15	33	95	90	58	94	81
32	11	04	25	94	88	50	92	77
33	10	0.2493	18	93	86	42	91	73
34	08	82	10	92	83	33	90	69

*If calibration of an apparatus shows a value of a significantly different from the 0.500 or 2.000 cc. in the column heading, the factors in the column are corrected by multiplying them by $\frac{a}{0.500}$ or $\frac{a}{2.000}$.

V. Determination of CO_2 in Blood or Plasma.

Introduction of Sample and Reagents.—The apparatus having been cleaned with dilute lactic acid as previously described, a drop of octyl alcohol is drawn into the capillary above cock *b*, and 2.3 cc. of CO_2-free water are put into the cup for each cc. of

blood or plasma to be added. Stop-cock *b* is closed, with *e* open and the leveling bulb at the level shown in Fig. 1. The blood sample is delivered beneath the layer of water in the cup from a pipette. Preferably a pipette with cock and rubber tip is used,

TABLE III.

Factors for Calculation of Millimols of Blood Gases per Liter from Pressures in 50 Cc. Apparatus.

Temperature.	Factors for CO_2.			Factors for O_2, CO, and N_2.				
	Sample =0.2 cc. S=2.0 " a=0.5 " i=1.03	Sample =1 cc. S=3.5 " a=2.0 " i=1.014	Sample =2 cc. S=7.0 " a=2.0 " i=1.014	Sample =0.2 cc. S=2.0 " a=0.5 " i=1.00	Sample=1 cc. S=3.5 "		Sample=2 cc. S=7 "	
					a=0.5 cc. i=1.00	a=2.0 cc. i=1.00	a=0.5 cc. i=1.00	a=2.0 cc. i=1.00
°C.								
15	0.1493	0.1216	0.0662	0.1388	0.02780	0.1113	0.01396	0.0558
16	85	11	56	84	70	09	90	56
17	80	04	51	80	61	05	85	54
18	73	0.1198	47	75	51	01	80	52
19	67	91	42	70	41	0.1097	75	50
20	61	85	38	65	31	93	70	48
21	54	78	34	60	21	89	65	46
22	46	72	29	55	11	85	60	44
23	39	66	25	50	02	81	55	42
24	33	61	21	45	0.02692	77	50	40
25	28	55	17	40	83	74	45	38
26	22	49	13	35	73	70	41	36
27	15	43	09	31	64	67	36	34
28	09	38	05	26	55	63	31	32
29	03	33	02	22	47	59	27	30
30	0.1398	28	0.0598	18	38	55	22	29
31	92	23	95	13	29	52	18	27
32	86	18	91	09	20	48	14	25
33	81	13	88	04	11	44	09	24
34	74	08	85	00	02	41	05	22

See foot-note to Table II.

as described under "Measurement of samples." If an ordinary pipette is used, the delivery is made with the pipette tip resting on the bottom of the cup, and during the delivery cock *b* is partially opened, so that most of the blood flows directly through *b*

into the chamber below, leaving a minimum amount accumulated in the cup to be washed in. After the delivery of the sample this residue of blood in the cup is run into the chamber below followed by the water layer, which washes the last traces of blood with it. Finally, 0.2 cc. of CO_2-free 1 N lactic acid per cc. of blood or plasma is added. The lactic acid may be conveniently measured by counting the drops; the drop number per cc. having been established. Stop-cock *b* is finally sealed with a drop of mercury.

Liberation of Carbon Dioxide.—The leveling bulb is lowered until the surface of the mercury (not of the water) has fallen to the *A* mark at the bottom of the chamber. Stop-cock *e* is then closed. The reaction mixture is shaken for 3 minutes.

Measurement of Pressure of Total Gases.—Mercury is readmitted by stop-cock *e* with the precautions previously discussed for CO_2 determinations under "Adjustment of gas volume," until the gas volume in the analysis pipette is reduced to 2.0 cc. Cock *e* is closed, the manometer is tapped with the finger, and the height of the mercury column is read (p_1 *mm.*).

Absorption of CO_2.—With blood, the variable amount of O_2 extracted with the CO_2 makes determination of the latter by absorption imperative. With plasma, although the air accompanying the CO_2 is sufficiently constant to be allowed for by calculation with fair accuracy, it is our custom to use absorption to determine the CO_2 when precision is desired.

The manner in which the aborbent alkali solution is added is important. The gas-free alkali solution (see "Gas-free reagents") must be run into the mixture of gases present without absorption of any gas other than CO_2. It was at first our custom before admitting the 1 N alkali, to open cock *e*, so that, with the leveling bulb at the level shown in Fig. 1, the pressure in the chamber rose to nearly atmospheric. This practice, however, permitted the absorption of enough oxygen by the gas-free alkali solution to cause, in whole blood analyses, an error of about + 0.2 volume per cent in the CO_2 determination.

To avoid this error, the 1 N NaOH solution is added under reduced pressure as follows. After measuring the pressure of the total extracted gas mixture the pressure is diminished so that the mercury level in the chamber is lowered somewhat below the position shown in Fig. 2, and a space of several cc. is left between the

water meniscus and the lower *a* mark. Cock *e* is then closed. 2 cc. of air-free 1 N NaOH are measured into the cup, with the delivering tip resting on the bottom of the cup so that the solution will not stream through and absorb air. 1 cc. of the alkali is then permitted to flow gradually through cock *b* into the chamber. Presumably because of the relatively uninterrupted motion of the gas molecules at the reduced pressure, absorption of CO_2 is extremely rapid. If 30 seconds are taken for running in the alkali we find absorption is complete.

That no measurable amounts of oxygen or air are absorbed with this technique we have proved by repeated comparisons of the manometer lowering (*c* values) caused by addition of 1.0 cc. of solution when the *a* space has been made free of all gases save water vapor, and also when it contained amounts of CO_2-free air greater than the amounts of oxygen obtained in blood analyses. The *c* values obtained in both cases were identical, indicating that no absorption of air occurred.

A simpler technique may be used for analyses of serum or water solutions of carbonates. The CO_2 may be absorbed with 0.2 cc. of 5 N NaOH measured with a pipette into the cup of the apparatus. This solution has solubility coefficients for O_2 and N_2 only one-tenth those of water, and may be used without being previously freed from air. It is added with the contents of the chamber at but slight negative pressure (cock *e* open, leveling bulb as in Fig. 1). When the CO_2 is absorbed the solution rises and washes the concentrated alkali out of the upper tube of the chamber with sufficient completeness to prevent error from vapor tension lowering. With whole blood, there is so much oxygen left in the top of the chamber that the blood solution does not rise high enough to rinse out the alkali; consequently, the technique described previously must be used, with air-freed 1 N NaOH added under diminished pressure.

Measurement of Pressure p_2 after Absorption of CO_2.—Absorption of CO_2 being completed, the solution in the chamber is lowered, if necessary, until its meniscus is a little below the *a* mark. The leveling bulb is placed in the position shown in Fig. 1, mercury is readmitted from cock *e* until the solution's meniscus is again on the *a* mark, and the p_2 reading is noted on the manometer. The CO_2 pressure P_{CO_2} is

$$P_{CO_2} = p_1 - p_2 - c$$

where c is the correction discussed on page 537. The CO_2 content of the solution is calculated from P_{CO_2} by multiplying with the factor obtained from Table II or III, or from a similar table derived from Equation 6 or 7. Cock b is sealed with mercury during the p_2 reading.

Sources of Error.—Aside from measurements of the sample and of the pressure and volume of the extracted gas there are certain minor sources of error, which, if not considered, may exert appreciable effect.

The ratio of unextracted gas to that extracted and measured is indicated by the term $\frac{S}{A - S}\alpha'$ in the equation by which the results are calculated. It is almost negligible when α' is as small (*viz.* 0.02 to 0.03) as in the cases of H_2, N_2, and O_2. At room temperature α'_{CO_2}, however, is about 1 (see Table I), so that when extraction has reached maximum completion about the same concentration of CO_2 exists per cc. of solution as per cc. of the supernatant gas phase. It is essential, consequently, that the value S, of total solution in the apparatus, be measured with an accuracy of about 0.05 cc. in order to keep errors from variation in S below 1 part per 1,000 in the final result.

Less accuracy in the measurement of A is required. In the 50 cc. apparatus an error of 1 cc. in A is required to introduce an error of 1 part per 1,000 in the final result.

A possible source of error may be encountered by the immediate analysis of samples chilled in the ice box, unless equilibration in the analysis chamber is continued for a time sufficient to allow the reaction mixture to reach the temperature recorded in the water jacket. *E.g.*, if the liquid in the chamber is at 19°C., and the water bath at 20°C., an error of 0.1 per cent is introduced into the calculated CO_2 content, chiefly because of the effect of temperature on the solubility coefficient of CO_2.

A constant slight error in the calculation of the CO_2 in blood and plasma analyses from the factors in Table I lies in the fact that the α' values used are those of pure water instead of the lower values of the diluted blood solution actually extracted. According to experiments by Van Slyke, Hastings, and Neill by the technique reported on page 568 of this paper, the solubility coefficients of the reaction mixtures used in plasma and blood analyses (1 cc. of blood or plasma + 2.5 cc. of water) are at 20°C., 0.850 and 0.838,

respectively, instead of 0.878, the value for water. The difference causes the CO_2 results calculated to be 1.5 parts per 1,000 too high for plasma, and 2 parts per 1,000 too high for whole blood. The effect is so slight, however, that it has not seemed to justify the compilation for this paper of additional tables for blood and plasma analyses.

Careful measurement of volume of the NaOH absorbent solution introduced is essential because of its effect on the *c* correction. In the different 50 cc. machines in use in our laboratory, with $S = 3.5$ cc., the value of correction *c* for addition of 1 cc. of alkali ranges between 2.0 and 4.0 mm. Consequently, an error of 0.1 cc. in the volume of the absorbent introduced in analysis of a 1 cc. blood sample, may result in an error of 0.4 mm. in the manometer reading, and an error of approximately 0.1 volume per cent of CO_2 in the results. For accuracy in measuring the alkali solution in the cup, mercury should just fill the capillary below the cup when the solution is placed in it.

The c correction is determined in a blank analysis in which *S* cc. (for 1 cc. of blood $S = 3.5$) of water, made alkaline with 2 or 3 drops of 1 N NaOH, are extracted in the apparatus, p_1 and p_2 being read before and after addition of 1 cc. of 1 N NaOH, or 0.2 cc. of 5 N NaOH, as above described.

$$c = p_1 - p_2$$

Determination of Correction i for Reabsorption of CO_2.—Factor *i* is determined by analysis of standard Na_2CO_3 solutions made up in CO_2-free water and protected from access of atmospheric CO_2. The value of *i* is calculated by rearrangement of Equation 7 in the form

$$(8) \qquad i = \frac{1}{P} \times \frac{[CO_2]\,(\text{cc. sample})}{0.0587\,a} \times \frac{1 + 0.00384\,t}{1 + \frac{S}{A - S}\alpha'}$$

where $[CO_2]$ = millimols of CO_2 per liter of the standard solution.

Na_2CO_3 was prepared by heating "reagent" $NaHCO_3$ with the precautions customary in preparing carbonate for the standardization of acids. Three different preparations of Na_2CO_3 were made

TABLE IV.

Estimated Corrections for Dissolved O_2 and N_2 in Blood.

Blood.	Determined.	Sought.	Correction to subtract.	
			vol. per cent	*mM per l.*
Venous	Total O_2	Combined O_2	0.1 (O_2).	0.04 (O_2).
Arterial	" "	" "	0.2 "	0.09 "
Saturated with air at 20°, 760 mm	" "	" "	0.5 "	0.22 "
Venous	Total $O_2 + N_2$.	" "	1.3 $O_2 + N_2$).	0.57 ($O_2 + N_2$).
Arterial	" " + "	" "	1.5 "	0.62 "
Saturated with air at 20°, 760 mm	" " + "	" "	1.9 "	0.85 "
Venous	" " + " or $CO + O_2 + N_2$.	Total O_2 or $CO + O_2$.	1.2 (N_2).	0.53 (N_2).
Arterial	" "	" " " " + "	1.2 "	0.53 "

from two different lots of $NaHCO_3$. The carbonate prepared was titrated against 0.1 N HCl which had been standardized gravimetrically. Two different solutions were made from each Na_2CO_3 preparation. As a check on the constancy of the i correction with different amounts of CO_2, solutions were prepared of 15, 30, and

TABLE V.

Determination of Reabsorption Correction i by Analysis of Standard Na_2CO_3 Solution.

A = 100.0 cc.; S = 5.00 cc.; a = 5.004 cc.; c = 1.5 mm. 2 cc. of standard Na_2CO_3 solution for each determination.

Date.	Na_2CO_3 preparation No.	Solution.		p_1	p_2	$P_{CO_2} = p_1 - p_2 - 1.5$	Temperature.	i^*
		No.	Concentration.					
1922			*mM*	*mm.*	*mm.*	*mm.*	*°C.*	
Nov. 27	B	B I	30.00	266.9	56.3	209.1	22.6	1.014
				266.0	55.9	208.6	22.6	1.016
	B	B II	29.91	267.2	57.5	208.2	22.5	1.014
				268.1	58.1	208.5	22.5	1.013
				266.0	57.0	207.5	21.4	1.013
Dec. 10	C	C I	48.85	397.9	53.0	343.4	24.7	1.015
				394.3	51.1	341.7	23.8	1.016
	C	C II	30.00	255.5	44.6	209.4	22.7	1.013
				254.4	43.6	209.3	22.5	1.012
Dec. 12	D	D I		248.6	37.9	209.2	22.5	1.013
				248.8	37.9	209.4	22.5	1.012
	D	D II	15.00	141.1	35.9	103.7	20.9	1.014
				140.1	34.9	103.7	20.6	1.012
Reagents				31.7	30.1	0.1		
Average								1.014

*From Equation 8.

45 millimolar concentration. Duplicate analyses were made on 2 cc. of each solution. Analyses were also made on different days as a further check on the constancy of the reabsorption correction. Results are given in Table V.

Since i is an empirical factor, it may vary slightly for different apparatus. It is also essential for the maintenance of the constancy of i that when the CO_2 volume is being reduced to a cc. after extraction, about the same time should be taken for the process in all analyses. If the time is materially prolonged i is somewhat increased.

Constancy of Results in CO_2 Determinations on Blood.—Duplicate analyses of 1 cc. samples of blood determined in the 50 cc. apparatus with $a = 2.0$ cc. usually agree within 0.2 volume per cent or 0.1 mM.

In experiments requiring the greatest precision we have used 2 or 3 cc. samples in the 100 cc. apparatus, with $a = 4$ or 5 cc. With 2 and 3 cc. samples our average variation in a series of duplicate analyses has invariably been below 0.05 mM. Table V represents a typical series of results with 2 cc. samples of blood and plasma saturated at different CO_2 tensions and determined in an apparatus whose $A = 100$ cc. and $a = 5.0$ cc.

VI. Determination of Plasma Carbon Dioxide Capacity.

The most complete information concerning the acid-base balance of the blood is obtained by measuring the CO_2 or $BHCO_3$ content of the blood as drawn, or preferably of the plasma, together with the plasma pH. With these two values, the state of the blood can be plotted on the acid-base chart (Van Slyke (1921)). However, the simple determination of the carbon dioxide capacity of the plasma by the original method of Van Slyke and Cullen (1917) is sufficient to reveal acidosis of metabolic origin, such as is encountered in diabetes and nephritis, and this procedure has such practical advantages that its use in many laboratories has been advisedly continued.

To perform the same determination with the present 50 cc. manometric apparatus one proceeds as described by Van Slyke and Cullen (1917) up to the point of the CO_2 determination. For this a drop of octyl alcohol is drawn into the capillary below the cup of the 50 cc. apparatus, and 1.5 cc. of 0.1 N lactic acid are measured into the cup. The 1 cc. sample of plasma, previously saturated with 5.5 per cent CO_2, is run into the cup under this layer of 0.1 N lactic acid. The lactic acid solution is run in after the plasma, and the chamber is evacuated and shaken 2 minutes.

The volume of gas is reduced to 2 cc. with the precautions described above in connection with CO_2 determinations, and p_1 is read on the manometer. The gas is then ejected, the ejected portion of solution is returned into the chamber, the cock is sealed with mercury, and the pressure is reduced until the gas space in the chamber is again 2 cc. The zero pressure p_2 is then read.

The pressure of the CO_2 plus the air extracted from the 2.5 cc. of solution is $p_1 - p_2$ mm. From the pressure thus obtained and

TABLE VI.

Duplicate Determinations of CO_2 in 2 Cc. Samples of Plasma.

$A = 100.0$ cc.; $S = 5.00$ cc.; $a = 5.004$ cc.; $i = 1.014$; $c = 1.5$ mm.

Sample No.	p_1	p_2	$P_{CO_2} = p_1 - p_2 - c$	Temperature.	CO_2 per l.
	mm.	*mm.*	*mm.*	*°C.*	*mM*
I	258.4	43.6	213.3	21.6	30.74
	258.9	43.8	213.6	21.7	30.79
II	236.3	43.6	191.2	21.7	27.57
	236.4	43.6	191.3	21.3	27.64
III	258.4	42.3	214.6	20.5	31.09
	257.5	41.9	214.1	20.5	31.04
IV	219.5	44.0	174.0	21.2	25.10
	219.9	44.0	174.4	21.9	25.10
V	198.2	45.8	150.9	22.3	21.69
	198.3	45.5	151.3	22.7	21.69

the temperature the CO_2 capacity of Van Slyke and Cullen is calculated graphically by means of the nomogram in Fig. 7.

Example. Initial manometer reading, p_1 = 257 mm.
Zero " " p_2 = 28 "
Pressure of extracted air and $CO_2 = p_1 - p_2$ = 229 "
Temperature = 21°C.

We lay a straight edge, or stretch a thread, across Fig. 7 from the point marked 229 mm. on the pressure scale to that marked 21° on the temperature scale. The straight line so drawn cuts the CO_2 capacity scale at 50.0 volumes per cent.

For CO_2 capacity determination as a measure of acidosis the original volumetric apparatus of Van Slyke (1917) is sufficiently

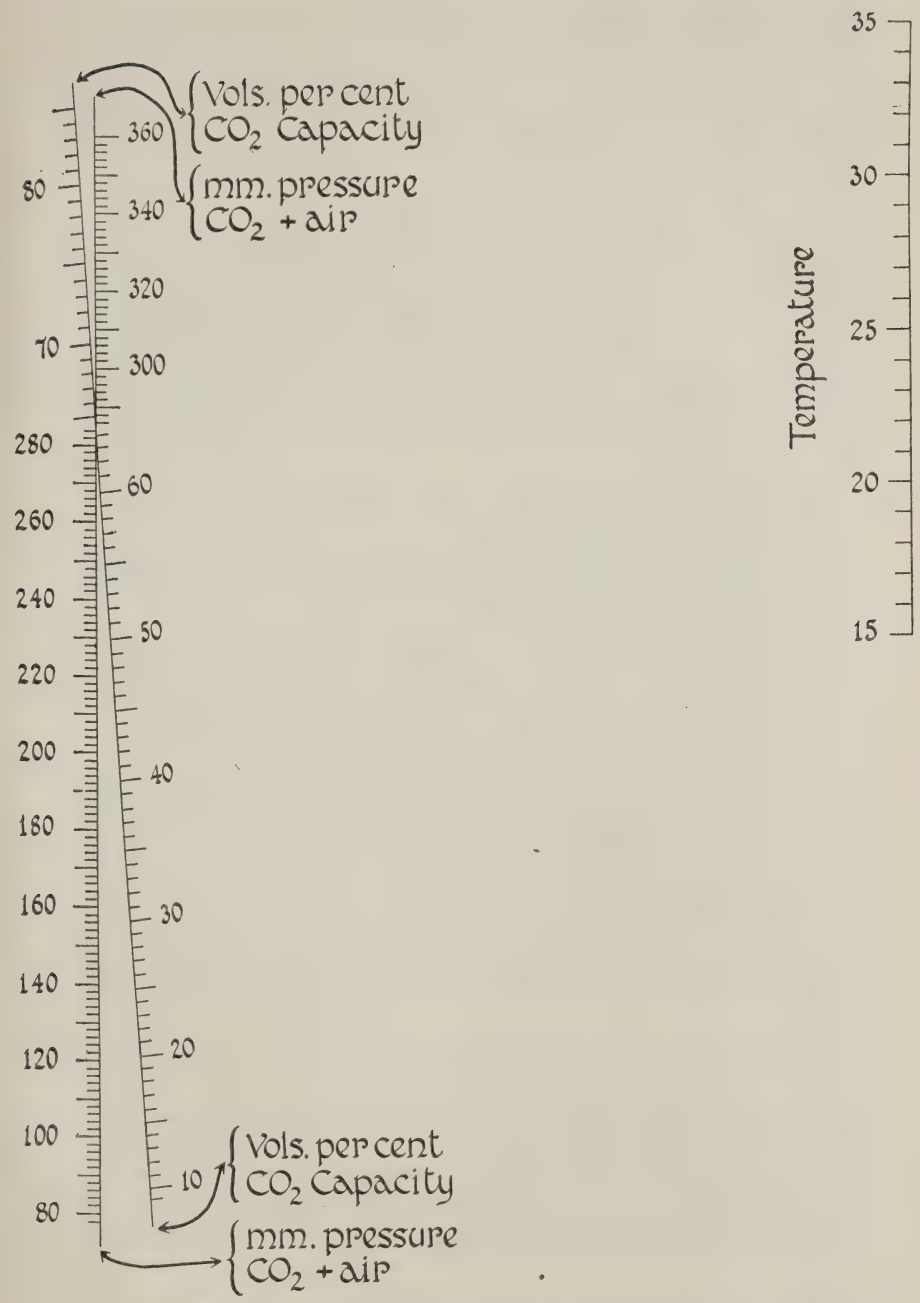

FIG. 7. Nomogram for calculation of plasma CO_2 capacity. A straight line connecting the observed points on the pressure and temperature scales cuts the CO_2 capacity scale at the point indicating the capacity.

accurate and is cheaper than the present constant volume apparatus with the manometer and shaker. The present apparatus, however, is equally convenient for the CO_2 capacity determinations, and can be used in addition for other purposes, described in this paper, some of which are outside the range of the original volumetric apparatus.

VII. Determination of Oxygen in Blood.

Analysis of 1 Cc. Samples of Blood.—Oxygen may be determined with the 50 cc. apparatus in samples of blood varying from 0.2 to 5.0 cc. 1 cc. samples enable one to obtain results with a variation in duplicates seldom exceeding 0.2 volume per cent. The alteration in technique when samples of 2.0 cc. are used will be described at the end of this section and those for 0.2 cc. samples will be noted later.

The principles used are those employed by Van Slyke and Stadie, with certain changes in detail.

The reagents for freeing the oxygen are prepared in a solution according to the following formula.

Potassium ferricyanide	3.0 gm.
Saponin (Merck)	3.0 "
Octyl alcohol	3.0 cc.
Water to	1,000 "

The saponin is several times the amount used by Van Slyke and Stadie, because the preparation now available has a relatively low hemolytic activity. Apparently the activity of different commercial preparations varies greatly. We have encountered none of which more was required than the amount provided by the above formula; but in case low results for oxygen should be obtained the factor which is the most likely to be responsible is the saponin.

The analysis is carried out as follows for a 1 cc. sample of blood:

The reagent mixture is stirred by rotation about the flask to obtain an emulsion of the octyl alcohol, and 7.5 cc. are measured into the apparatus (from the 2 cc. mark in the chamber to the 5.5 cc. mark in the cup). The air is removed from the solution by evacuating the apparatus and shaking for 3 minutes. During the

shaking the surface of the mercury should be in the tube at the bottom of the chamber, and about 1 cm. below the point at which the tube broadens into the 50 cc. bulb. Thus located, the mercury remains fairly quiet, and has minimum contact with the reagent solution. The ferricyanide reacts with the mercury at a rate which is insignificant under these conditions, but might destroy a considerable part of the ferricyanide if the mercury were whirled about the broader part of the chamber and thus brought into intimate contact with the solution. A slight trace of air still remains in solution, calculable by the solubility coefficient, but it is included in the *c* correction described below.

During the de-aeration of the reagent solution the sample of thoroughly mixed blood is drawn into a 1 cc. pipette, preferably provided with a stop-cock and rubber tip, as described on page 532, and shown in Fig. 4.

The 3 minute extraction of the reagents being complete, 6 cc. of the solution are forced up into the cup, leaving 1.5 cc. in the chamber. The sample is at once run under the liquid in the cup into the chamber, as described previously under "Measurement of samples." The pipette is then withdrawn with the least possible agitation of the reagent solution in the cup, and 1 cc. of the latter is permitted to flow into the chamber, rinsing through the blood in the capillary, and leaving 5.0 cc. in the cup. The cock is now sealed with a drop of mercury and the 5 cc. of solution in the cup are discarded. The purpose of the discarded upper 5 cc. of solution is to protect from air the lower 1 cc. which is returned to the chamber. Van Slyke and Stadie did not use such a protecting layer, and neglected the error caused by reabsorption of air in the reagent solution in the cup. This error, however, would be well within the limits of measurement in the present apparatus, as shown by Table VII, if all the solution from the cup were returned to the chamber.

The apparatus, with the blood and reagents in the chamber, is evacuated and shaken 3 minutes. 1 cc. of air-free 1 N NaOH is placed in the cup and the CO_2 is absorbed by admitting 0.5 cc. of the hydroxide into the chamber under diminished pressure, by the technique described above for CO_2 determinations on whole blood. The value of p_1, indicating the pressure of the $O_2 + N_2$ is then determined with the meniscus of the solution at the

2.0 cc. a mark, unless the O_2 content is low because of anemia or unusually low saturation of the hemoglobin, in which case the reading at $a = 0.5$ cc. is preferable.

One may now either determine the O_2 directly by absorption with hydrosulfite or may measure the $O_2 + N_2$ together and correct for the known, nearly constant N_2 content of blood.

Measurement of O_2 by Absorption.—When maximum precision is desired, or when the blood contains CO, the O_2 is determined by absorption with hydrosulfite. After the CO_2 has been removed and the p_1 reading recorded, cock e is opened, with the leveling bulb in the position shown in Fig. 1, so that the gas in the apparatus is under but slight negative pressure. 1 cc. of hydrosulfite

TABLE VII.

Successive Determinations of O_2 and N_2 in 2 Cc. Samples of Horse Blood Saturated with Air at 21°.

No.	P_{O_2} ($a = 2.0$ cc.)	P_{N_2} ($a = 0.5$ cc.)	Temperature.	O_2 content.	N_2 content.
	mm.	*mm.*	*°C.*	*vol. per cent*	*vol. per cent*
1	173.8	45.4	24.0	21.03	1.38
2	174.5	44.3	24.0	21.12	1.34
3	174.3	43.2	24.2	21.09	1.32
4	174.7	43.6	24.5	21.11	1.32
5	174.3	44.0	24.5	21.07	1.33

solution (see "Gas-free reagents") is introduced into the cup, and 0.50 cc. is admitted as follows. Cock b is turned until a small drop has passed through and down the walls of the tube below. At intervals of about 5 seconds, the rest of the 0.50 cc. is admitted in small drops. In between 2 and 3 minutes the 0.50 cc. of solution is admitted and all the gas absorbed except a small bubble of nitrogen (unless CO is present). The solubility of N_2 in the hydrosulfite solution is only about one-fourth as great as in water. Consequently the absorption of O_2 may be performed at atmospheric pressure without measurable N_2 absorption. The leveling bulb is now lowered, the gas volume is again brought to the same volume as before, 2.0 or 0.5 cc., and p_2 is read on the manometer.

$$P_{O_2} = p_1 - p_2 - c_{O_2}$$

The value of c_{O_2}, when 0.50 cc. of hydrosulfite is used, is in the usual 50 cc. apparatus 1.5 to 2.0 mm., and is determinable within 0.1 mm. for a given apparatus.

As a check on the completeness of the absorption of the O_2 and the avoidance of error from incomplete removal of atmospheric air from the reagents, one may now eject the N_2 and measure p_3, in the gas-free apparatus.

$$P_{N_2} = p_2 - p_3 - c_{N_2}$$

The N_2 should be approximately 1.2 volumes per cent for blood drawn from the body or saturated with air at 38°, and 1.4 volumes per cent for blood saturated with air at 20°.

A convenient and complete alternative oxygen absorption with hydrosulfite may be obtained by adding 1.0 cc. of the hydrosulfite solution, lowering the mercury until the mixture of diluted blood and hydrosulfite is at the bottom of the chamber, and then shaking for 2 minutes. The drawback to this mode of absorption is that if any traces of dissolved air have been admitted with either alkali or the hydrosulfite solution they will pass into the gas phase and will cause a lowered O_2 and an increased N_2 result.

In place of hydrosulfite, pyrogallol may be used for absorption of O_2. It forms a somewhat gummy precipitate on the wall of the chamber at the point where it flows down into contact with the hemoglobin solution. For this reason it must be run into the chamber while the latter is partly evacuated, so that the contact between pyrogallol and the blood solution occurs in the broad part of the chamber well below the *a* mark. The pyrogallol is accordingly added under diminished pressure in the manner described for use with 1 N NaOH in CO_2 determinations. Absorption with a small volume of pyrogallol is slower than with hydrosulfite. 1 cc. of pyrogallol is admitted in small drops, at about 10 second intervals; the process must continue over 4 or 5 minutes to insure complete absorption. The pressure is then allowed to rise to atmospheric, in order that the solution may rise and wash out the pyrogallol from the upper tube of the chamber. Otherwise the effect of the concentrated alkali on the vapor tension may lower the p_2 reading by 2 or 3 mm. The p_2 reading is finally taken. The

c correction with 1 cc. of pyrogallol is naturally about twice as great as when the absorption is done with 0.50 cc. of hydrosulfite. The gummy precipitate referred to redissolves when the apparatus is rinsed out with water.

Pyrogallol is not so rapid, clean, or convenient as hydrosulfite. Its advantage over the latter is that the pyrogallol solution is stable. It may be used, therefore, when only an occasional analysis is performed. If more than one oxygen determination is to be made the writers prefer to expend the few minutes required to make up a fresh hydrosulfite solution.

For analysis of 2 cc. of blood the technique is the same as for 1 cc. samples except that 10 cc. instead of 7.5 cc. of ferricyanide-saponin solution are taken at the start, 5 cc. being discarded as in the above procedure.

The constancy of results attainable is indicated by Table VII.

Measurement of O_2 + N_2 Together.—In this case, after p_1 has been read, the O_2 and N_2 are ejected from the chamber, and p_2 is determined with the meniscus of the solution at the same a mark. The O_2 is calculated by subtracting the N_2, or N_2 + dissolved O_2, as indicated in Table IV.

Determination of c Correction for Oxygen Determinations.—When oxygen is determined by absorption with hydrosulfite or pyrogallol the c correction in the calculation $P_{O_2} = p_1 - p_2 - c_{O_2}$ is the sum of two small corrections. One, of about 1.5 mm., is due to the lowering of the mercury meniscus in the chamber caused by adding the 0.5 cc. of hydrosulfite, and another of about 0.2 mm. at $a = 2$ cc., or 0.8 mm. at $a = 0.5$ cc., is due to a slight amount of oxygen left in the reagent solution when the latter is extracted once as described.

The value of c is determined by extracting 7.5 cc. of reagent solution for 3 minutes, and ejecting 5 cc. of the mixture, 2.5 cc. being left in the chamber, as in the analysis. This solution is shaken 3 minutes, and then 1.5 cc. of air-free 1 N NaOH are run in, in the manner previously described. p_1 is read with the gas volume at both 0.5 and 2.0 cc. The hydrosulfite is then run in, as in the analysis, and the readings are repeated, giving p_2 at both points.

$$c_{O_2} = p^1 - p_2$$

Values for c_{O_2} are thus obtained which may be used when pressures are read with gas volumes of either 0.5 or 2.0 cc.

Finally, the residual N_2 is ejected and the reading p_3 is taken.

$$c_{N_2} = p_2 - p_3$$

If O_2 and N_2 are determined together the value of c is obtained in the same manner, except that p_2 is measured after ejection of the O_2 and N_2 together without a preliminary absorption of O_2. The ejected gas bubble may be too small to see readily, but its pressure is measurable.

Corrections for Dissolved O_2 and N_2 of Blood in Calculation of Total or Combined O_2.—The estimated corrections for dissolved O_2 and N_2 are given in Table IV. The N_2 dissolved by blood is taken as 1.2 volumes per cent for circulating blood, and 1.4 for blood saturated with air at 20°, 760 mm. These figures, the average obtained by the present technique, are slightly lower than the average found by Van Slyke and Stadie. The dissolved O_2 values in Table IV are estimated on the assumption that the solubility of uncombined oxygen in blood is proportional to the water content of the latter. The exactness of this assumption is uncertain, as the difficulty of distinguishing between dissolved and combined oxygen has thus far prevented direct determinations of the solubility of the gas in blood. All the corrections given in Table IV are approximate estimates, and must vary somewhat with the cell content of the blood.

Diffusion of Air into the Extracted Reagent Solution in the Cup of the Apparatus.—During the stay of the reagent solution in the cup it is exposed to air which rapidly diffuses downwards into it. For this reason only the bottom 1 cc. of the 6 cc. in the cup is returned to the chamber with the blood. The extent of air diffusion into the dilute aqueous solution in the cup is indicated by the following experiment.

12 cc. portions of the ferricyanide-saponin reagent solution were rendered quite air-free by two successive extractions of 2 minutes each. 6 cc. were then run up into the cup and allowed to stand for measured intervals. Varying amounts were returned to the chamber and the air dissolved during the exposure was extracted and measured. The results are given in Table VIII.

Van Slyke and Stadie returned the entire 6 cc. of reagent solution in their analyses. In 2 minutes, which is about the usual period through which this

air-freed solution stands in the cup during an analysis, the data of Table VIII indicate that about 0.0035 cc. of atmospheric air is absorbed, of which from the relative O_2 and N_2 concentrations and solubilities, one-third is presumably oxygen. In analyses of a 2 cc. sample of blood, as directed by

TABLE VIII.

Air Diffusing into Successive Layers of 6 Cc. of Reagent Solution During Exposure in the Cup of the Apparatus.

Period of exposure standing in cup.	Volume of solution returned from bottom of cup to chamber.	Pressure of air measured at 0.5 cc. volume and 20° after extraction.	Volume of air at 0°, 760 mm.	Estimated volume of oxygen.*
min.	*cc.*	*mm.*	*cc.*	*cc.*
2	1	0.6	0.0004	0.0001
2	1	0.7	04	01
2	2	1.9	11	04
2	2	2.0	12	04
2	3	2.3	14	05
2	3	2.4	14	05
2	4	2.6	16	05
2	4	2.9	17	06
2	5	5.0	30	10
2	5	4.0	24	08
2	6	5.8	35	12
2	6	5.9	35	12
5	6	10.8	65	22
5	6	10.2	61	20
0†	6	3.8	22	07
0	6	4.8	28	09

*Dissolved oxygen estimated to be one-third the dissolved air, as in water saturated with air.

†Solution run up into cup and returned at once.

Van Slyke and Stadie, the error introduced would, therefore, be about + 0.06 volume per cent of oxygen, when the latter was determined by absorption, or + 0.18 volume per cent when it was determined by subtraction of the estimated N_2 content of blood.

When only the bottom 1 cc. is returned, as in the technique outlined in the present paper, this error from absorption of atmospheric oxygen is reduced to about 0.01 volume per cent of a 1 cc. sample.

VIII. Combined Determination of Oxygen and Carbon Dioxide in Blood.

The procedure up to the point of absorbing the gases and measuring the pressures is the same as that described for oxygen determination in 1 cc. of blood, except that to 1 liter of the reagent saponin-ferricyanide solution, 40 cc. of 1 N lactic acid are added, so that in the final blood-reagent mixture approximately 0.1 cc. of 1 N lactic acid will be present for each cc. of blood.[4]

When the blood is run down into the acid ferricyanide solution in the chamber of the apparatus, a precipitate forms, as described by Van Slyke and Stadie. As soon as cock *b* has been sealed with a drop of mercury, the leveling bulb of the apparatus is raised and lowered two or three times, so that the solution and precipitate in the top of the chamber are drawn down into the enlarged lower part and then permitted to rise back to the cock again. This procedure detaches the precipitate from the wall and breaks it up into fine granules, which remain suspended in the solution and do not interfere with any subsequent steps in the analysis.

The level of the mercury is lowered to the 50 cc. mark, and the apparatus is shaken 3 minutes.

The gas volume is then reduced to 2 cc., with the precautions already described for CO_2 determinations. The pressure reading p_1 is then taken.

For absorption of the CO_2 with 1 N NaOH the pressure is lowered somewhat, as already described for this stage of CO_2 determinations, so that the gas volume is increased to about 5 cc. Then 0.5 cc. of air-free 1 N NaOH is admitted. As the alkali mixes with the blood solution the precipitate previously mentioned dissolves. The mixing of blood and alkali is completed by moving the chamber back and forth two or three times with the hand. Vigorous shaking of the alkaline solution is to be avoided, or

[4] Van Slyke and Stadie (1921), p. 33.

reabsorption of O_2, noted by Van Slyke and Stadie, may occur. Finally, the gas volume is restored to 2 cc. and pressure p_2 is read.

$$P_{CO_2} = p_1 - p_2 - c_{CO_2}$$

After the p_2 reading the oxygen is absorbed with 0.5 cc. of hydrosulfite solution, as already described for oxygen determinations; p_3 is then read on the manometer.

$$P_{O_2} = p_2 - p_3 - c_{O_2}$$

If N_2 is also to be determined, a p_4 reading is taken with a gas volume of 0.5 cc., the residual gas is ejected and the pressure is reduced in the gas-free chamber till the solution meniscus is again on the 0.5 cc. mark. p_5 is then read.

$$P_{N_2} = p_4 - p_5 - p_{N_2}$$

The N_2 cannot, of course, be determined in this manner if CO is present.

If the oxygen content of the blood is low, the above procedure may be changed by reading the oxygen pressure with a 0.5 cc. instead of a 2.0 cc. volume.

IX. Determination of Carbon Monoxide in Blood.

To determine carbon monoxide in blood the latter is treated with an acidified ferricyanide solution. The CO_2, O_2, CO, and N_2 are extracted together. The CO_2 and O_2 are absorbed with alkaline hydrosulfite, and the CO is determined by subtracting from the CO + N_2 the average N_2 value, 1.2 volumes per cent.

With the neutral ferricyanide solution previously described for oxygen determinations it is impossible to extract all the CO within a reasonable time. If 0.1 cc. of 1 N lactic acid per cc. of blood is added, however, and the ferricyanide is somewhat increased above that required for HbO_2 decomposition, HbCO may be decomposed and the CO extracted in 2 minutes. The acid solution also sets free the CO_2, which may be determined with the O_2 and CO.

The reagent solution for blood containing CO is accordingly made as follows:

Saponin	3.0 gm.
$K_3Fe(CN)_6$	8.0 "
1 N lactic acid	40.0 cc.
Caprylic alcohol	3.0 "
Water to	1,000 "

For the analysis 2 cc. of blood are preferably taken. 10 cc. of the reagent solution are freed of air, as described for O_2 determination in 2 cc. of blood, and the 2 cc. of blood plus 5 cc. of air-free reagent solution are extracted for 3 minutes in the evacuated apparatus.

The CO_2 and O_2 are absorbed by addition of 1.0 cc. of an alkaline solution of hydrosulfite. For this purpose the hydrosulfite is made up in 1.0 N KOH instead of the 0.5 N KOH ordinarily used. The hydrosulfite is added in the manner previously described for O_2 determinations, in small portions during 3 or 4 minutes with slight negative pressure.

At this point a precaution is required because of the high affinity between CO and reduced hemoglobin. As Conant (1923) has shown, hydrosulfite reduces methemoglobin to reduced hemoglobin, which can recombine with O_2 or CO to form HbO_2 or HbCO. The reduction occurs as soon as the hydrosulfite mixes with the blood solution, and can be seen by the change from the dark methemoglobin color to the clear red of reduced hemoglobin. As soon as the reduced hemoglobin has been formed in the solution the latter begins to reabsorb the CO that has been extracted from it. If unnecessary agitation is avoided, however, the amount reabsorbed is, under the conditions of the analysis, approximately constant at 2.4 per cent of the total CO present. (Since this factor is empirical, it is well for the analyst to redetermine it with his own apparatus and technique, by analyzing some blood saturated with illuminating gas. The percentage of the total CO absorbed when hydrosulfite is run in, as described below, is used as correction instead of 2.4 if found to be different.)

The hydrosulfite is run into the chamber without agitation of the solution (the leveling bulb is allowed to rest quietly in the position shown in Fig. 1, cock *e* being open). As soon as the absorption of O_2 is complete, cock *e* is closed and the leveling bulb is placed in the lowest position. Cock *e* is then gradually opened, and the level of the fluid in the chamber is quietly lowered

to either the 0.5 or the 2 cc. mark, according to whether little or much CO is present. Care is taken not to overrun the mark, as the manipulation of the solution to bring it back would increase the amount of CO absorbed. The liquid level in the chamber should be lowered at a sufficiently slow rate also, to permit complete drainage of the solution down the walls.

The pressure p_1 is read. It represents the combined pressure of CO and N_2.

The gas is then ejected from the chamber and pressure p_2 is read. The CO + N_2 pressure is then calculated as

$$P_{CO + N_2} = 1.024\ (p_1 - p_2)$$

From the $P_{CO + N_2}$ the CO + N_2 content is calculated according to Table II or III, and 1.2 volumes per cent are subtracted for the N_2.

$$\text{Vol. per cent CO} = \text{Vol. per cent (CO} + N_2) - 1.2$$

X. Combined Determination of CO_2, O_2, and CO.

The determination is performed like the simple CO estimation, except that the CO_2 is absorbed separately with NaOH.

After extraction of the total gases is completed their volume is reduced to 2 cc., with the precautions described for CO_2 determinations, and p_1 is read.

The CO_2 is obtained by absorption with 1.0 cc. of 1 N NaOH under diminished pressure, in the manner described for CO_2 determinations. The pressure p_2 is measured at 2 cc. volume.

The oxygen is then absorbed with 1.0 cc. of hydrosulfite as described above. Pressure p_3 is read. The residual CO + N_2 is ejected, and final pressure p_4 is read, also at 2 cc. gas volume.

Indicating the drop in pressure after absorption of CO_2 with NaOH as $\Delta P_{NaOH} = p_1 - p_2 - c$ and similarly the pressure reduction corrected for c, after absorption of hydrosulfite as $\Delta P_{Na_2S_2O_4}$ and the reduction after ejection of the residual CO_2 and N_2 as $\Delta P_{ejection}$, we calculate as follows:

$$CO_2 \text{ content} = \Delta P_{NaOH} \times f_{CO_2}$$

$$O_2 \text{ content} = (\Delta P_{Na_2S_2O_4} - 0.024\,\Delta P_{ejection}) \times f_{O_2}$$

$$CO \text{ content} = (1.024\,\Delta P_{ejection}) \times f_{CO} - 1.2$$

where f_{CO_2} and f_{O_2} are the factors of Table II or III. Within the limits of experimental error, as mentioned before, the f_{O_2} values may be employed for f_{CO}.

The results of some analyses are given in Table IX.

TABLE IX.

Determination of CO_2, O_2, and CO in 2 Cc. Samples of Blood.

a = 2.00 cc. for all pressure readings.

Blood.	ΔP after NaOH.	ΔP after $Na_2S_2O_4$.	ΔP after ejection.	Temperature.	CO_2 content.	O_2 content.	CO content.	N_2 content.
	mm.	*mm.*	*mm.*	*°C.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
A.	*	162.4	9.5	19.2		20.00	0.00	1.15
Saturated	*	162.6	9.0	20.0		19.98	0.00	1.09
with air	230.8	162.1	10.1	20.0	32.51	19.92	0.00	1.29
at 38°.	232.3	161.8	8.7	20.0	32.73	19.88	0.00	1.08
Average					32.62	19.94		1.14
B.	126.4	3.8	159.5	20.6	17.76	0.00	18.82	(1.2)
Saturated	129.5	3.9	161.5	21.0	18.15	0.00	19.05	(1.2)
with N_2	128.1	4.1	162.5	20.5	18.01	0.02	19.18	(1.2)
+ 10 per cent CO at 38°.								
Average					17.95	0.01	19.02	
Mixture of	178.2	81.4	84.8	20.3	25.08	9.75	9.45	(1.2)
equal volumes of *A*	176.7	82.7	86.1	21.0	24.73	9.88	9.59	(1.2)
and *B*.								
Average					25.90	9.81	9.52	
Calculated mean of *A* and *B*					25.29	9.94	9.51	

*Oxygen was freed with neutral ferricyanide, hence no CO_2 results were determined.

XI. Micro Analyses.

Analyses of 0.2 Cc. Samples of Blood with the 50 Cc. Apparatus.— The following reagents are used for freeing the gases.

For CO_2, 0.01 N lactic acid.

For O_2, the same ferricyanide solution described for larger samples on page 554.

For combined CO_2 and O_2, the same ferricyanide solution plus lactic acid to 0.01 N concentration.

For the determination, 6 cc. of the proper reagent solution are measured into the apparatus and freed from gases by extracting for 3 minutes. 5 cc. of the solution are run up into the cup, the 0.2 cc. sample of blood is run under the layer of solution in the cup and thence into the chamber of the apparatus, together with sufficient of the reagent solution to bring the total volume of fluid in the apparatus to the 2 cc. mark.

The apparatus is evacuated in the usual manner and shaken for 3 minutes.

The gas pressures are all measured at 0.5 cc. volume.

For absorbing CO_2 5 N NaOH is used; for absorbing O_2, the usual 20 per cent hydrosulfite described under "Gas-free reagents." Of either solution, 0.1 cc. is measured from a pipette into the cup, which should be entirely free of droplets of other solutions. There should be just enough mercury above cock *b* to fill the capillary. With the chamber of the apparatus under slight negative pressure (leveling bulb as in Fig. 1) the absorbing solution is admitted in 3 or 4 droplets, about 10 seconds being allowed to elapse after each admission. When the solution has been run in so far that just enough remains above the cock to fill the capillary some mercury is poured into the cup. One makes certain that no air bubble is trapped between the mercury and the hydroxide or hydrosulfite solution, and then proceeds with the admission of the remainder of the solution. The mercury follows it and seals the cock.

The factors for calculating CO_2 and O_2, respectively, from the observed pressure are given in the second and fifth columns of Tables II and III. The correction factor *i* for reabsorption of CO_2 is greater when the volume is decreased to 0.5 cc. for measurement than when the latter is taken with the gas volume at 2 cc. With the apparatus we have used the percentage absorbed is 3.0 in the former case as compared with 1.4 in the latter. Consequently in Tables II and III the *i* factor for the micro analyses of CO_2 is given as 1.03. While great variations of *i* from this value are improbable, it is desirable, when maximum accuracy is desired, to determine *i* in the apparatus used by analyses of standard Na_2CO_3 solutions.

For each apparatus blank analyses are to be performed in order to determine the *c* correction. With 0.1 cc. of absorbent solution added the correction is about 1 mm.

Analyses of 0.2 Cc. Samples of Blood with the 10 Cc. Apparatus.—When the micro analysis is used as a routine, it is convenient to use an apparatus with a chamber of 10 cc. volume, and one *a* mark at 0.4 cc. The calibrated tube at the top of the chamber should be between 3 and 4 mm. inner diameter. For the rest, the micro chamber is proportioned like the 50 cc. one shown in Figs. 1 and 2. The cup is graduated above the capillary in 0.1 cc. divisions to hold 1.5 cc.

The reagents for freeing the gases are the following.

For CO_2, 0.017 N lactic acid, made by diluting 1 cc. of 1 N acid to 60 cc.

For O_2, the same neutral ferricyanide solution previously described.

For combined CO_2 and O_2, the same ferricyanide solution plus 1 cc. of 1 N lactic acid per 60 cc.

1.5 cc. of the reagents are accurately measured into the apparatus, extracted 3 minutes to free from gases, and 0.6 cc. is run up into the cup. The blood sample is run under this solution and is followed into the chamber by the latter until only 0.3 cc. remains in the cup above the capillary. A drop of mercury is then added, and the rest of the analysis is carried out in the usual manner. Absorption of CO_2 is performed with 0.1 cc. of 5 N NaOH, absorption of O_2 with 0.1 cc. of 20 per cent hydrosulfite, as described above.

For calculation of the results the factors used are obtained by multiplying by 2 those of Tables II and III in the columns headed "Sample = 2 cc., S = 7.0 cc., a = 2.0 cc." The relative proportions of S, a, and A are the same, all being one-fifth those in the 50 cc. apparatus, but the sample is one-tenth as great, so that the factors must be doubled.

The *pipettes used for micro analyses* have been of the Ostwald type, with bulbs holding slightly less than 0.2 cc., and stems of 0.6 to 0.7 mm. bore and 3 mm. external diameter. We have determined the bores of the capillary tubes, before using them to make pipettes, by weighing mercury columns of measured length in the tubes. The tips of the pipettes are not constricted, but are tapered by grinding them down on a fine carborundum wheel. The pipettes are calibrated to deliver 0.2 cc. between two marks,

the lower of which should be at least 6 cm. above the tip. Blood allowed to flow freely from pipettes made thus will be delivered at a rate sufficiently slow for satisfactory drainage.

The results of micro analyses in both 50 and 10 cc. chambers are given in Tables X and XI.

XII. Determination of Gases in Liquids Saturated at High Tensions.

The technique already outlined for measuring samples and transferring them to the apparatus limits the exposure of the sample to air sufficiently to prevent measurable loss of CO_2 or gain of oxygen when the tensions in the sample are within or near the physiological range. When the gas tensions are high, however, more rigid precautions are required. The technique outlined below was devised for determining the CO_2 contents of solutions saturated with the pure gas at atmospheric pressure, and yielded results constant with $\pm$ 0.2 per cent of the values determined.

For measuring the samples a 3-way, 120 degree stop-cock was sealed to the stem of an Ostwald pipette which was calibrated to deliver 5 cc. from the lower mark, up to, but not including, the capillary of the stop-cock (Fig. 8). One arm (*h*) leading from the stop-cock was bent downward the other arm (*g*) was bent upward in such a manner that a smooth joint could be obtained with the capillary extension of the upper stop-cock of the apparatus (Fig. 9). A 2-way stop-cock was sealed to the lower stem of the pipette below the calibration mark. A mercury leveling bulb was attached to the lower stem of the pipette by means of rubber tubing.

The saturated CO_2 solution was kept over mercury in a Barcroft tube. In order to draw a sample for analysis the 5 cc. pipette was connected with the Barcroft tube as shown in Fig. 8. By proper manipulation of cocks and leveling bulbs *h* was filled with mercury from the pipette then *h* and *g* were washed with a few drops of the solution from the Barcroft tube. The sample was then drawn into the pipette as far as the 5 cc. mark on the lower stem. While the sample was being drawn, the leveling bulb of the Barcroft tube was placed at its maximum height, and the connecting cocks were all entirely open, the rate of flow being regulated by the lower cock of the pipette, in order that the

TABLE X.

Determination of CO_2, O_2, and N_2 in 1 and 0.2 Cc. Samples of Blood with the 50 Cc. Apparatus.

Sample.	Apparatus.	P_{CO_2}	P_{O_2}	P_{N_2}	Temperature.	CO_2 content.	O_2 content.	N_2 content.
cc.		*mm.* ($a = 2.0$)	*mm.* ($a = 0.5$)	*mm.* ($a = 0.5$)	*°C.*	*vol. per cent*	*vol. per cent*	*vol. per cent*
1	I*	194.5	226.2		23	51.0	13.7	
1	I	193.9	221.1	17.4	23	50.8	13.5	1.1
1	II*	194.6	230.3	20.3	23	51.1	13.8	1.2
1	II	194.9	228.4	21.0	23	51.0	13.6	1.3
		($a = 0.5$)	($a = 0.5$)	($a = 0.5$)				
0.2	I	157.0	44.8	4.1	23	50.7	13.6	1.2
0.2	I	157.8	46.2	5.1	23	51.0	14.0	1.5
0.2	I	160.0	45.2	4.3	23	51.7	13.7	1.3
0.2	II	160.7	45.3	4.7	23	50.9	13.5	1.4
0.2	II	161.7	46.0	6.2	23	51.3	13.7	1.8
0.2	II	157.6	46.1	4.5	23	50.0	13.7	1.3

*The a volumes found by calibration of the two chambers were as follows:

Apparatus.	Volume at 2 cc. mark.	Volume at 0.5 cc. mark.
	cc.	*cc.*
I	2.005	0.500
II	2.006	0.490

The factors are accordingly smaller for Apparatus II at the 0.5 cc. a mark than for Apparatus I.

TABLE XI.

Determination of CO_2, O_2, and N_2 in 1 Cc. Samples with the 50 Cc. Apparatus and in 0.2 Cc. Samples with the 10 Cc. Apparatus.

Sample.	P_{CO_2}	P_{O_2}	P_{N_2}	Temperature.	CO_2 content.	O_2 content.	N_2 content.
cc.	*mm.* ($a = 2.0$)	*mm.* ($a = 2.0$)	*mm.* ($a = 0.5$)	*°C.*	*vol. per cent*	*vol. per cent*	*vol. per cent*
1	158.2	79.3	17.8	21.0	41.78	19.36	1.10
1	159.0	80.1	19.4	21.0	41.98	19.55	1.19
	($a = 0.4$)	($a = 0.4$)	($a = 0.4$)				
0.2	142.7	78.1	4.5	18.7	41.2	19.3	1.1
0.2	145.4	78.7	4.6	19.0	41.8	19.4	1.1
0.2	146.0	79.8	5.3	19.0	42.0	19.7	1.3
0.2	145.0	77.9	4.8	19.0	41.7	19.2	1.2

sample should be continually under more than atmospheric pressure.

With the pipette cocks as shown in Fig. 9 (but before connection with the gas analysis chamber), tubes *g* and *h* were washed with water and dried by suction. The pipette was then connected with the gas analysis chamber as shown in Fig. 9, and tubes *g* and *h* were filled with mercury from the chamber. The

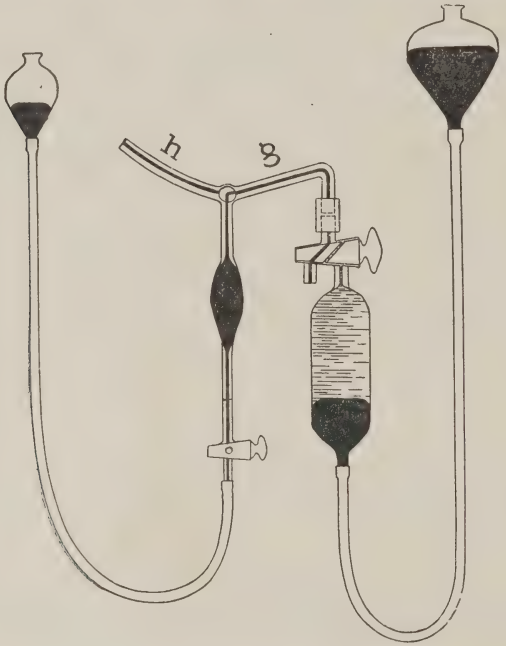

FIG. 8. Pipette for measuring samples of solutions entirely protected from contact with air. Pipette is shown connected with Barcroft tube containing solution, from which a sample is about to be drawn.

upper cock of the pipette was then turned to permit the sample to flow over into the chamber. The transfer was made with a slow, regular flow, to insure a constant completeness of drainage. The cocks were kept open until the mercury from the pipette had followed the solution through *g* into the analysis chamber and sealed cock *b*.

The analysis was carried out as previously described for CO_2 determinations.

The same technique apparently would be convenient for the analysis of solutions of other gases dissolved under high tensions, or of solutions from which for other reasons rigid exclusion of contact with the atmosphere is imperative.

XIII. Determination of Dissolved Gases in Water.

For determination of the dissolved gases in water we have used 10 cc. samples, which are run into the apparatus without other solutions save a few drops of 1 N acid to free CO_2 from any car-

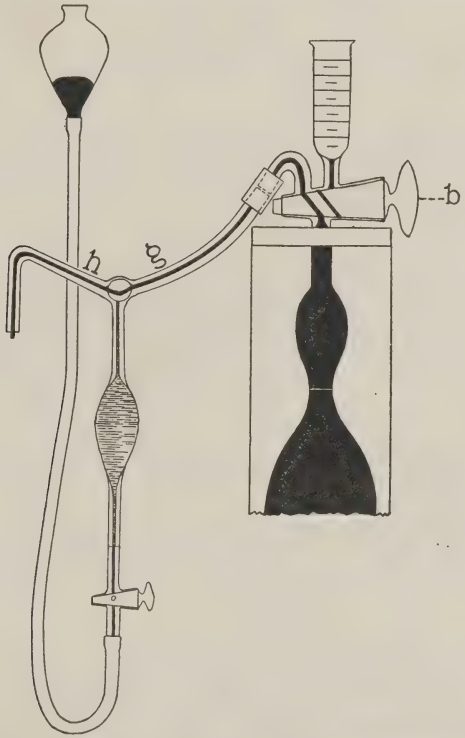

FIG. 9. Transfer of sample from pipette to extracting chamber of apparatus.

bonate that may be present. The results in Table XII indicate the degree of constancy obtainable. The factors used were calculated from Equation 6.

In the last two analyses, samples of only 2 cc. were used, and the determinations were performed as in analyses of 2 cc. samples of blood. The results indicate that the technique used prevents significant error from contamination of the reagents with air.

XIV. Use of the Apparatus for Air and General Gas Analysis.

The apparatus can be used for a convenient and fairly accurate gas analysis. It is not in the present form devised to yield results as accurate as those by the Haldane apparatus, but we have found it convenient and satisfactory in analyzing gas mixtures with CO_2 or O_2 contents outside the range of the Haldane apparatus.

The apparatus is washed with acidified water in the manner previously described (p. 534). The zero point, p_0, is measured

TABLE XII.

Gas Content of Water Saturated with Air at 21°, 760 Mm.

No.	Water.	Sample.	P_{CO_2} ($a = 0.5$)	P_{O_2} ($a = 0.5$)	P_{N_2} ($a = 0.5$)	Temperature.	CO_2 content.	O_2 content.	N_2 content.
		cc.	*mm.*	*mm.*	*mm.*	*°C.*	*vol. per cent*	*vol. per cent*	*vol. per cent*
1	Distilled.	10.00	33.5	105.3	192.5	20.0	0.276	0.650	1.182
2	"	10.00	32.2	108.3	191.5	20.0	0.265	0.669	1.176
3	" + drop HCl.	10.00	7.9	103.3	193.5	20.0	0.065	0.638	1.188
4	" + " "	10.00	8.6	102.6	193.3	20.0	0.071	0.633	1.187
5	" + " "	2.00		17.8	39.8	24.0		0.59	1.22
6	" + " "	2.00		20.5	38.8	24.0		0.68	1.19
	Found by Winkler for water saturated at 21° (Landolt-Börnstein's tables)							0.623	1.211

The factors for the 10 cc. samples, as calculated by Equation 6 for 20°, are $f_{CO_2} = 0.00825$, $f_{O_2} = 0.00617$, $f_{N_2} = 0.00614$. The factors for the 2 cc. samples are those of Table II.

on the manometer with the mercury meniscus in the chamber at the 2 cc. mark. The gas sample is drawn into the chamber through the capillary side tube of cock *b*. Sufficient gas is admitted to give nearly an atmosphere of pressure at 2 cc. volume in the 50 cc. apparatus, or at 4 cc. volume in the 100 cc. apparatus, which is preferable. p_1 is then measured on the manometer.

To absorb the CO_2 exactly 1 cc. of air-free 1 N NaOH is run into the chamber. The mercury is lowered to the 50 cc. mark, and

the solution is shaken 1 minute to absorb the CO_2. The gas volume is brought back to 2 cc. and p_2 is measured.

Similarly, to absorb O_2, 1 cc. of air-free hydrosulfite solution is introduced, and is shaken for 2 minutes. p_3 is then read on the manometer.

The c corrections for the effect of the added 1 cc. portions of alkali and hydrosulfite are found in blank determinations in the gas-free apparatus. The results are calculated as follows:

$$\text{Per cent } CO_2 = \frac{p_1 - p_2 - c}{p_1 - p_0}$$

$$\text{Per cent } O_2 = \frac{p_2 - p_3 - c}{p_1 - p_0}$$

For this calculation based on pressure changes only, it is assumed that during the few minutes required for the analysis the temperature in the water jacket remains constant within 0.1°C.

Results by the above method check within usually 0.1 volume per cent those by the Haldane apparatus. They are somewhat more consistent if obtained with the 100 cc. apparatus, with pressure measurements at 4 cc. of gas volume, than with the 50 cc. apparatus.

The methods here presented were in part developed with the technical assistance of John Plazin, who constructed the first apparatus and performed many of the analyses with which the details of technique were developed.

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ON THE DETERMINATION OF GASES IN BLOOD AND OTHER SOLUTIONS BY VACUUM EXTRACTION AND MANOMETRIC MEASUREMENT. II.

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INTRODUCTION.

The extraction chamber of the blood gas apparatus described in the preceding paper by Van Slyke and Neill (1) is simplified, in comparison with the original constant pressure apparatus (Van Slyke (2)) by omission of the lower stop-cock and the small chamber beneath it, previously used for removing solutions from the chamber as a preliminary to gas readings. With the apparatus thus simplified it is possible to determine by direct absorption both CO_2 and O_2, since both the absorbents used (NaOH solution and hydrosulfite, respectively) can be added while the diluted blood is still in the chamber without diminishing the accuracy of the determination. In the determination of CO, however, with absorption of this gas by CuCl solution, the latter forms such a precipitate with the alkaline solutions previously added that it is necessary to remove them. Carbon monoxide can be determined in the Van Slyke-Neill apparatus only by measuring the residual CO + N_2, and subtracting the average N_2 content of blood. For determination of CO by absorption it is necessary to remove the extracted solution and the subsequently added gas absorbents from the chamber before the gas readings are made. Also in determining the O_2 obtained by the reaction of $KMnO_4$ on standardized H_2O_2 solutions, as a check on the blood oxygen method, it was found desirable to remove the reagents before the readings were made.

For these purposes the somewhat less simple apparatus and technique described in the present paper have been used, the lower cock and drainage chamber, in a modified form, being restored.

With this apparatus, and a relatively non-absorbent solution of equal volumes of saturated NaCl and glycerol to wash out the extracted blood and reagents, it has furthermore been found possible to perform CO_2 determinations on blood and standard carbonate solutions without correction for reabsorption, and thereby obtain an additional check on the accuracy of the procedure and factors used.

Description of Modified Extraction Chamber.

It will be seen from Fig. 1 that the chamber is similar to that described by Van Slyke and Neill except for the trapping arrangement at the lower end. This consists of a large 3-way stop-cock (*a*) of 3 mm. bore which is fused on to the bottom of the chamber. Below the stop-cock is attached the trap which consists simply of a bulb (*b*) of about 15 cc. capacity. The stop-cock is so arranged that it is possible to place the trap in communication either with the gas chamber or with the outlet (*c*). By lowering the mercury leveling bulb the contents of the chamber may be drawn down into the trap, and by reversing the stop-cock and raising the mercury they may be expelled through the outlet (*c*).

The stop-cock, (*a*), and the tubes connecting it with the chamber and bulb are all conveniently of 3 mm. bore. The stop-cock (*a*) and adjacent parts should be heavily built in order to stand the strain during mechanical shaking, which is increased by the slight eccentricity of the apparatus at this point.

In using the apparatus it is, of course, important to avoid drawing any of the gases into the trap with the reagents; if desired the lower stop-cock may be sealed between each trapping and expulsion by means of mercury introduced from above, but with a well ground cock this step is not necessary.

The apparatus is shown with a simple 2-way stop-cock connecting the upper end of the pipette with the cup. A 3-way cock is unnecessary because the washing out can be done through the outlet from the lower stop-cock.

In use the extraction chamber, like the Van Slyke-Neill chamber, is surrounded by a water jacket which extends between the upper and lower stop-cocks.

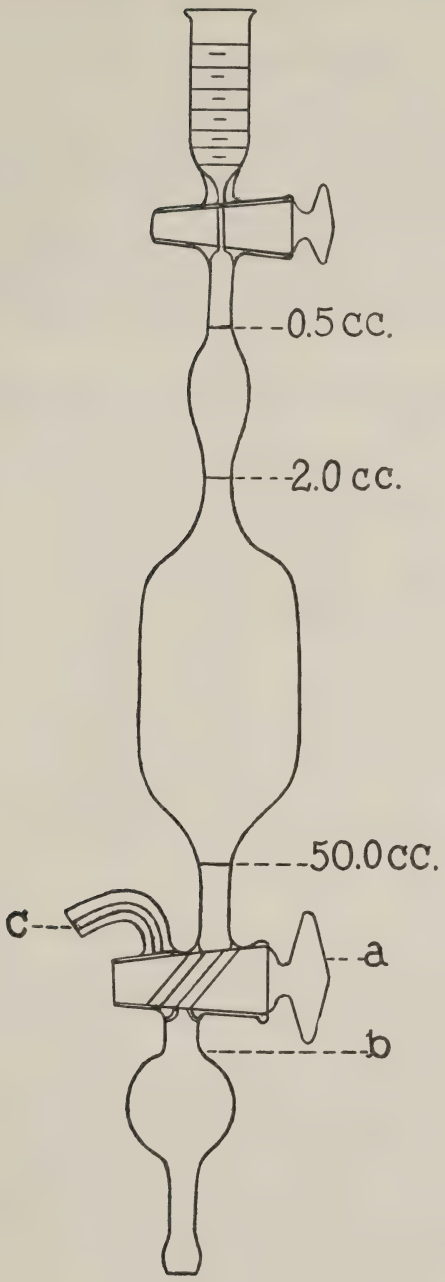

Fig. 1.

Determination of Carbon Dioxide.

In the determination of carbon dioxide the sample and the reagent are introduced into the machine precisely as described by Van Slyke and Neill. After extraction of the carbon dioxide the reagent mixture is drawn into the trap and expelled, and into the empty machine are introduced 3 cc. of the air-free glycerol-salt solution. The pressure of gas is then read (p_1) and 0.2 cc. of 5 N sodium hydroxide solution is introduced to absorb the CO_2. The

TABLE I.

Gasometric Determinations of Pure Na_2CO_3 Solutions.

No.	Concentration of Na_2CO_3 by weight.	Gasometric determination of CO_2.	
		1 cc. sample.	0.2 cc. sample.
	mM per l.	*mM per l.*	*mM per l.*
1	5.00	4.95	
		4.97	
2	10.00	9.96	
		10.00	
		9.96	
3	15.00	14.96	
		14.97	
		15.00	
4	30.00	29.96	29.70
		29.96	
		30.02	29.62
5	45.00	45.30	
		45.12	44.97
		45.10	44.95
		45.10	44.70

Solutions made up from freshly prepared sodium carbonate and redistilled water which was boiled out immediately before use.

alkaline solution is replaced by 3 cc. of fresh glycerol-salt solution and the pressure of the residual gases (p_2) measured. $P_{CO_2} = p_1 - p_2$. The carbon dioxide present is then estimated by multiplying P_{CO_2} by the appropriate factor for the apparatus employed and the temperature at the time of analysis. The factors are calculated from the data in Table I of Van Slyke and Neill, without the correction factor i.

In Table I are shown the results of analyses of a series of solutions of sodium carbonate of known strength. It will be seen that in the case of the 1 cc. sample the agreement between the observed and theoretical concentrations of carbon dioxide is good throughout. The analyses of samples of 0.2 cc. show more variability, but this is due to error in the measurement of the sample rather than in the method.

The above description of technique applies equally to the analysis of blood and of pure carbonate solution.

TABLE II.

Gasometric and Titrimetric Determination of H_2O_2 Solutions.

No.	Gasometric O_2.			$KMnO_4$ titration.		
	(*a*)	(*b*)	Mean.	(*a*)	(*b*)	Mean.
	*m*M	*m*M		*m*M	*m*M	
1	38.18	38.20	38.19	38.16	38.20	38.18
2	33.25	33.28	33.27	33.08	33.14	33.10
3	26.82	26.70	26.76	26.75	26.71	26.73
4	31.46	31.50	31.48	31.44	31.48	31.46
5	27.18	26.98	27.09	27.02	27.08	27.05
6	6.23	6.20	6.22	6.26	6.21	6.24

Solutions of H_2O_2 evacuated by mechanical pump to remove dissolved oxygen and sample taken for titration with potassium permanganate immediately after the gasometric determination.

Determination of Labile Oxygen in Standard H_2O_2 Solutions and in Blood.

The solutions which we employed as standard for our oxygen determinations were prepared as follows: a 3 per cent solution of pure hydrogen peroxide was diluted with distilled water to the approximate concentration desired; the diluted solution was then transferred to a paraffined suction flask and evacuated by means of a mechanical pump to a pressure of about 1 mm. of mercury to remove dissolved oxygen. Immediately after evacuation the flask was filled with carbon dioxide and kept tightly corked; in this way it was possible to keep the solution practically oxygen-free during the period of an experiment.

The analysis was carried out as follows: 2.5 cc. of dilute sulfuric acid were shaken air-free in the apparatus and run up into the cup; 1 cc. of the peroxide solution was then run into the machine

under the air-free sulfuric acid. When all the sulfuric acid and peroxide were in the machine, 2 drops of saturated potassium permanganate were introduced, to decompose the hydrogen peroxide. The mixture was drawn down to the bottom of the gas pipette and shaken for 3 minutes. At the end of this period the reagent mixture was drawn off through the lower stop-cock into the trap and expelled from the machine. The carbon dioxide was then absorbed by means of 1 cc. of air-free normal sodium hydroxide or by a little concentrated potassium hydroxide, either of which was introduced into the machine when the mercury was still lowered to the bottom of the pipette and was subsequently drawn off and expelled. There were now introduced into the machine 3 cc. (carefully measured in the cup) of an air-free solution consisting of equal parts of glycerol and saturated sodium chloride, which had been mixed and rendered air-free as described by Van Slyke and Neill (see Fig. 6 of their paper). This solution has such a low solubility for the gases (about one-fifth that of water) that under the conditions used it does not absorb them to a measurable extent. The pressure p_1 of gas was then read at the constant volume mark (2 cc. or 0.5 cc. as convenient). Then the glycerol-salt solution was lowered into the trap and expelled from the machine, the mercury was run up, and about 2 cc. of hydrosulfite or pyrogallol were introduced. With pyrogallol, absorption was accelerated by repeatedly raising and lowering the leveling bulb. After absorption was complete the solution was drawn down into the trap and expelled. With the mercury held at the bottom of the pipette, about 5 cc. of glycerol-salt solution were allowed to flow in from the cup, washing down the sides of the pipette. These washings were drawn off and expelled. 3 cc. of glycerol-salt solution (carefully measured) were introduced into the machine and the pressure, p_2, read again with the gas at the constant volume mark. The calculation of the result from the reading $p_1 - p_2$ is exactly the same as in the case of the Van Slyke-Neill machine of similar dimensions, but the correction, c, is here unnecessary.

The reason for introducing the glycerol-salt solution in the above methods instead of taking the readings over the mercury itself is that it is not possible to drain the pipette completely, and, when the mercury is run up into the narrow part of the pipette, small

differences in the amount of liquid above the surface of the mercury cause considerable variation in the p reading. With 3 cc. of liquid, however, the surface of the mercury is brought down into the wide part of the pipette where a small error in the measurement of this 3 cc. would make no appreciable difference in the mercury level.

The hydrosulfite or pyrogallol used need not be kept within a small definite volume, since it is replaced by glycerol-salt solution before the reading is taken. The absorption of the oxygen in this method should not take longer than 1 minute and the complete analysis about 20 minutes.

In Table II will be found in the left-hand column a series of duplicate gasometric determinations of solutions of hydrogen peroxide by the method described above, and in the right-hand column are the results of titrations of these same solutions by means of potassium permanganate performed immediately after the gas analysis. It will be seen that agreement is obtained over a wide range of concentration.

The foregoing description applies exactly to the determination of blood oxygen with the necessary modifications of the reagents employed, which are described by Van Slyke and Neill.

Determination of Carbon Monoxide in Blood.

The extraction of the O_2 and CO from the mixture of blood and acidified ferricyanide solution is performed as described by Van Slyke and Neill, and the oxygen is absorbed, *after* removal of the blood solution, as in the oxygen determination described above in this paper. The blood is removed from the apparatus and the latter washed once with glycerol-salt solution before the hydrosulfite or pyrogallol is added, so that reabsorption of CO by reduced hemoglobin does not occur.

The residual gas consists of CO and N_2. The CO is absorbed by adding, with slight negative pressure, 3 cc. of Winkler's cuprous chloride solution (200 gm. of CuCl, 250 gm. of NH_4Cl, 750 cc. of water), which has been rendered air-free as described in the preceding paper.

The combination of CO and CuCl is a loose, reversible union. Consequently, if, after the absorption, the absorbing solution is lowered in almost a complete vacuum to the bottom of the chamber

and drawn off into the trap below, as is the hydrosulfite or pyrogallol after oxygen absorption, some of the CO will bubble out of the solution and return to the gas phase. Three or four successive portions of the cuprous chloride absorbent must be added and removed in this manner before all the CO is removed from the chamber.

A simpler method is, after CO absorption is complete, merely to lower the solution till its meniscus is on the 0.5 or 2.0 cc. *a* mark and measure the pressure. This can be done without measurable return of CO to the gas phase. A *c* correction, as in Van Slyke and Neill's calculations, is necessary here, because the cuprous chloride has about 2 mm. higher vapor tension than the glycerol-salt solution. Hence

$$P_{CO} = p_1 - p_2 + c$$

The value of *c* is determined empirically by control experiments in which pressure readings in the gas-free apparatus are made alternately with 3 cc. portions present of glycerol-salt solution and of the cuprous chloride solution, respectively, *c* representing the difference between the readings. It is to be noted that the *c* correction in this case is added to the $p_1 - p_2$ value instead of being subtracted from it, as it is in all the calculations used with the Van Slyke-Neill apparatus.

The N_2 may be determined by ejecting the gas left after absorption with cuprous chloride, and measuring the residual pressure over the cuprous chloride solution.

Combined Determination of CO_2, O_2, and CO in the Same Sample of Blood.

Either 1 or 2 cc. samples may be used. All three gases are freed together by the acid ferricyanide reagent solution described by Van Slyke and Neill for CO determination. After extraction the successive reductions in gas pressure caused by absorption with 5 N NaOH, hydrosulfite, and cuprous chloride solutions are measured as described in the preceding sections. Each pressure measurement is taken with the gas over 3 cc. of glycerol-salt solution, except the reading after CO absorption. This is taken more conveniently over the cuprous chloride solution, for

reasons given above in the description of the carbon monoxide determination.

It is advisable to perform control determinations with the reagents alone, chiefly to make certain that no air is introduced

TABLE III.

Analyses of Blood.

No.	O_2	CO_2	Combined method, 1 cc. sample.	
	2 cc. sample.	1 cc. sample.	O_2	CO_2
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*
1	12.49	21.12	12.44	20.92
	12.50	21.10	12.40	20.90
2	9.12	19.21	9.03	19.25
	9.06	19.24	8.94	19.41
3	5.26	25.92	5.26	25.90
	5.19	25.96	5.26	25.84

TABLE IV.

Determination of O_2 and CO in 1 Cc. of Blood.

Blood.	P_{O_2}	P_{CO}	Temperature.	O_2 content.	CO content.
	mm. ($a = 0.5$)	*mm.* ($a = 0.5$)	*°C.*	*vol. per cent*	*vol. per cent*
A.	365.5		22.2	22.22	
Saturated with air at 38°.	367.9		22.2	22.37	
Average				22.30	
B.		387.2	22.8		23.47
Saturated with CO.		286.7	22.8		23.42
Average					23.45
C.	189.5	191.4	21.4	11.53	11.64
Mixture of equal volumes of *A* and *B*.	188.2	192.8	23.4	11.38	11.66
	188.4	191.5	25.3	11.33	11.52
Average				11.41	11.61
Average 1/2 *A* and 1/2 *B*				11.15	11.72

with them. If pressure differences are found in the control analyses, they are to be used as corrections to the P_{CO_2}, P_{O_2}, P_{CO}, and P_{N_2} obtained in the blood analyses.

In Table III are results of a series of experiments which were carried out to test the accuracy of this combined method of estimation in oxygenated blood. On the same specimen of blood, which was kept in a closed tube over mercury, there were carried out the following estimations; (*a*) duplicate oxygen analyses on 2 cc. samples; (*b*) duplicate carbon dioxide determinations on 1 cc. samples; and (*c*) duplicate determinations of both gases on the same sample (1 cc.) by the method just described. The results shown in Table III of three such experiments indicate the accuracy obtainable by this method.

In Table IV are given the results obtained by analyzing separately oxygenated and carbon monoxide blood (saturated with illuminating gas), respectively, and a mixture of equal parts of each blood.

SUMMARY.

A modified form of the extraction chamber used with the manometric apparatus is described which permits the removal of successively added reagents before each gas measurement.

The technique is described for determining with the apparatus CO_2, O_2, CO, and N_2, either separately or all in one sample of blood. The reabsorption corrections for CO_2 and CO are eliminated.

The accuracy of the oxygen determination has been demonstrated by simultaneous gasometric and titrimetric analyses of solutions of hydrogen peroxide.

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ANTIKETOGENESIS.

V. THE KETOLYTIC REACTION; ACTION OF GLYCOL ALDEHYDE AND OF GLYOXAL.*

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In an earlier paper (1) a reaction was reported by which glucose, when undergoing oxidation by hydrogen peroxide in alkaline solution, accomplishes the simultaneous oxidation of acetoacetic acid. This "ketolytic" action of glucose appears to be a reaction of fundamental biochemical importance, because of its probable relation both to the phenomenon of the avoidance of ketosis, "antiketogenesis," and to the question of the pathway of the oxidation of glucose in the animal body.

One of the writers has pointed out (2–6) that the well known "antiketogenic" action of carbohydrate in avoiding or abolishing ketosis in the human body is explained by the hypothesis that it is due to a chemical reaction between an oxidation product of glucose and acetoacetic acid, the product being readily oxidized,—in contrast with free acetoacetic acid which resists oxidation, and for this reason accumulates unless it reacts with the "ketolytic" derivative of glucose. According to this conception ketosis is, therefore, the result of the production of acetoacetic acid from its ketogenic precursors in larger amounts than corresponds with the amount of "ketolytic" substance being formed at the same time from the oxidation of glucose. The analogy between the ketolytic reaction *in vitro* and the phenomenon of antiketogenesis in the

* The data in this paper represent a part of a dissertation presented by T. E. Friedemann for the degree of Doctor of Philosophy, Washington University, 1923.

body is so close that there can be little doubt that a knowledge of the former will go far to explain the mechanism of the physiological reaction.

It is *only during oxidation* that glucose exerts its antiketogenic or "ketolytic" effect in *both* reactions. This fact makes it probable that *the active ketolytic derivative of glucose in vitro is a stage upon the pathway of glucose oxidation in the animal body*, and may

TABLE I.

Comparison of Rate of Oxidation of Glucose and Acetoacetate, Separately and Together, by H_2O_2 in 0.5 N KOH at ± 30°C.

Experiment No.	66	64 *a*	64 *b*	65 *a*	65 *b*	64 *a*	64 *b*
	mMols	*mMols*	*mMols*	*mMols*	*mMols*	*mMols*	*mMols*
Added per liter.							
Glucose	0	20	20	20	20	20	20
Acetoacetate	20	20	17.8	0	0	20	17.8
H_2O_2	200	200	200	200	200	200	200
Time.	Acetoacetate consumed.			Total acid formed.			
hrs.	*mMols* (1)	*mMols* (2)	*mMols* (3)	*cc.* N (4)	*cc.* N (5)	*cc.* N (6)	*cc.* N (7)
0.25				2.4	1.0	6.9	2.7
0.50	0.7	1.5	0.4				
1.00	0.7	3.2	3.9	25.7	24.2	31.5	34.3
2.00	2.1	12.5	10.73				
3.00				81.4	79.3	165.4	150.0
4.00	3.4	18.1	16.2	90.2	89.1	173.7	164.6
5.00	(4.0)*	(18.2)*	(16.3)*	94.3	93.3	175.4	166.3
6.00	4.4	18.3	16.4				166.4
27.50	10.5						

* Figures in parentheses are interpolated from curves in Chart 1.

thus indicate by what route glucose is oxidized, a question about which knowledge is yet very meager and uncertain. These considerations lead us to suppose that the "ketolytic reaction" of the simple sugars is of fundamental importance in relation to these intricate problems of intermediary metabolism; and a detailed knowledge of the reaction is, therefore, highly desirable.

From this point of view we have undertaken a study of the ketolytic reaction, a report of which is contained in this and later papers.

When either glucose alone (or other monosaccharide) or acetoacetic acid alone is added to an alkaline solution of hydrogen peroxide, each is oxidized at a rate which may be accurately measured by titrating the acids formed, or by determining the rate of disappearance of the sugar or acetoacetate. The sugars are oxidized rapidly; the acetoacetate quite slowly. But if both sugar and acetoacetate are present in the same solution and are thus undergoing oxidation together, the rate of disappearance of acetoacetate is enormously accelerated, with a corresponding increase in the rate of acid production. *The rate of acetoacetate disappearance becomes equal to and parallels the rate of sugar oxidation;* that is to say, the *rate of keto acid consumption, or "ketolysis," is determined and limited by the rate of sugar oxidation.* This fact is demonstrated by the following set of experiments, the data of which are given in Table I, and shown as curves in Charts 1 and 2. Glucose alone (20 mMols), potassium acetoacetate alone (20 mMols), and both together, were simultaneously added to an excess of KOH (final concentration 0.5 N) and H_2O_2 (200 mMols), and each solution was diluted to 1 liter. The temperature when mixed was about 30°C. and remained approximately constant. At intervals, portions of the solutions were withdrawn and analyzed for acetoacetate, and titrated for total acid formed (decrease of alkalinity + alkali equivalent of consumed acetoacetate). It will be seen from the results in Table I that the oxidation of acetoacetate alone is slow, being in this case only about half complete in 27 hours, as compared with about 90 per cent complete in 4 hours when oxidized in the presence of glucose. These results are plotted in Chart 1.

The amount and rate of acid formed by oxidation of glucose + acetoacetate likewise exceed that from glucose alone. (Columns 6 and 7 compared with 4 and 5.) The rate of oxidation of glucose alone (as indicated by its rate of acid production), may be brought to a basis of comparison with the rate of acetoacetate disappearance and of acid production when both are oxidized together, by expressing each in percentage of the total at completion. Taking the values after 5 hours to represent the end of the reactions, and calculating as percentages of these values the acid production from glucose alone (Nos. 65*a* and 65*b*), and the acetoacetate consumption

(Nos. 64 *a* and 64 *b*), we have the data of Table II and the curve shown in Chart 2. All the points fall on or near a single curve,

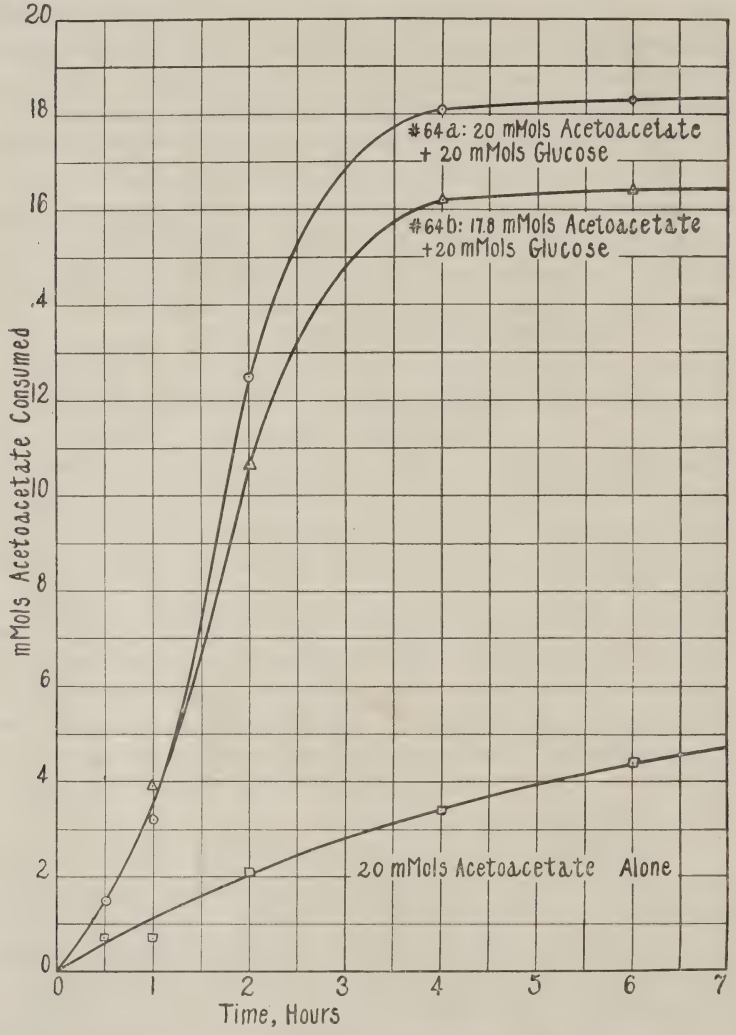


CHART 1. Rate of acetoacetate consumption in 0.5 N KOH + H_2O_2 with and without glucose. Temperature approximately 30°C.

though there is the suggestion that the rates of acetoacetate consumption and of acid production from the ketolysis are more rapid

TABLE II.

Comparison of Rate of Oxidation of Glucose and Acetoacetate by H_2O_2 in 0.5 N KOH at ± 30°C.

Experiment No.	65 *a*		65 *b*		64 *a*		64 *b*		64 *a*		64 *b*	
	Glucose alone.				Glucose + acetoacetate.				Glucose + acetoacetate.			
	Acid production.				Acid production.				Acetoacetate consumption.			
Time, *hrs.*	*cc.* N	*per cent**	*cc.* N	*per cent**	*cc.* N	*per cent**	*cc.* N	*per cent**	*mMols*	*per cent**	*mMols*	*per cent**
0.25	2.4	2.5	1.0	1.0	6.9	3.9	2.7	1.6				
0.50									1.5	8.2	0.4	2.4
1.00	25.7	27.2	24.2	25.9	31.5	17.9	34.3	20.6	3.2	17.6	3.9	23.9
2.00									12.5	68.7	10.7	65.6
3.00	81.4	86.4	79.3	85.0	165.4	94.2	150.0	90.2				
4.00	90.2	95.7	89.1	95.6	173.7	99.1	164.6	99.0	18.1	99.4	16.2	99.4
5.00	94.3	100	93.3	100	175.4	100	166.3	100	18.2	100	16.3	100

* Per cent of acid production (or acetoacetate consumption) after 5 hours.

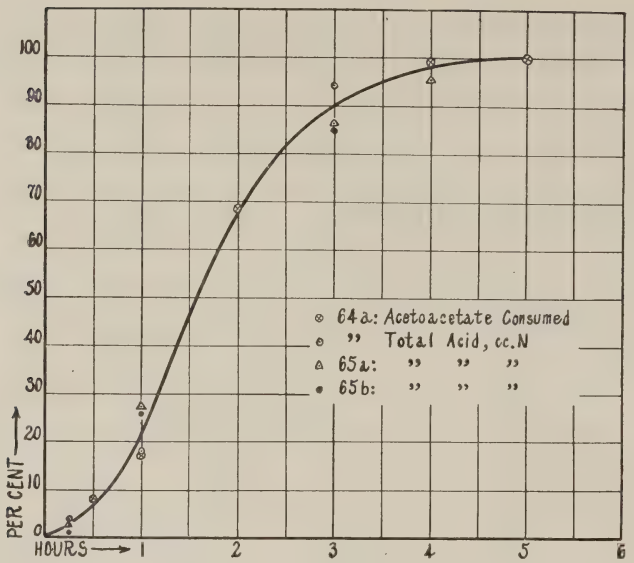


CHART 2. Rate of acid production from oxidation of glucose alone (Nos. 65 *a* and 65 *b*), and of glucose + acetoacetate (No. 64 *a*) compared to the rate of acetoacetate consumption. All data are expressed in percentage of the total after 5 hours. See Tables I and II.

TABLE III.

Effect of Varying Amounts of Glucose upon Rate of Acetoacetate Oxidation by H_2O_2 in 0.5 N KOH at 38°C.

Experiment No.	46	47	48	54	49	50	51
	mMols	*mMols*	*mMols*	*mMols*	*mMols*	*mMols*	*mMols*
Added per liter.							
Acetoacetate	18.1	18.1	18.1	18.4	18.1	18.1	18.1
Glucose	0	5	10	20	40	80	120
H_2O_2	200	200	200	200	200	200	200
Time.	Acetoacetate consumed per liter.						
hrs.	*mMols*	*mMols*	*mMols*	*mMols*	*mMols*	*mMols*	*mMols*
0.25					0.1	0	0
0.50				0.7	2.1	2.8	3.7
0.75					4.2	7.5	8.5
1.0				2.7	7.8	12.7	14.7
2.0		4.2	6.7	10.2	16.2	16.7	16.7
4.0	1.6	7.8	12.0	16.9	16.8	H_2O_2 exhausted, and browning of solution at 3 hrs.	
6.0	3.0	9.2	13.6		17.0		

toward the end than the rate of glucose oxidation alone. At any rate, it is quite evident that the rate of acetoacetate consumption is at least as fast as, and is apparently determined by, the rate of glucose oxidation.

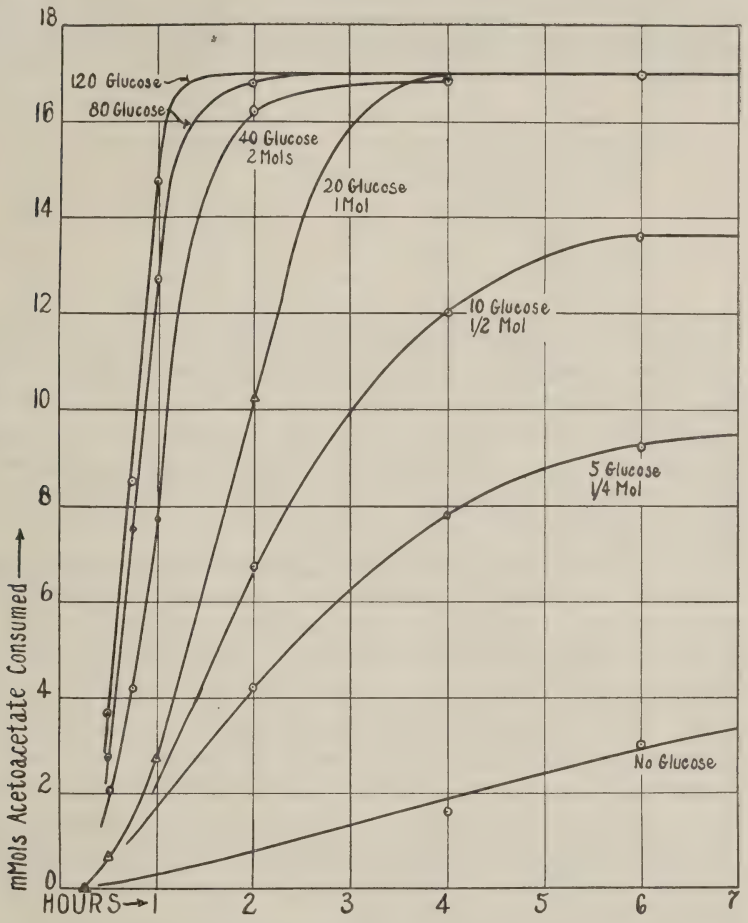


Chart 3. Effect of varying amounts of glucose on the rate of acetoacetate oxidation in 0.5 N KOH at 38°C.

In Table III and Chart 3 are given data showing the effect of varying amounts of glucose upon the rate of total consumption of acetoacetic acid. About 18 mMols per liter of acetoacetate in 0.5 N KOH were oxidized at 38°C. by H_2O_2 in the presence of 0,

0.25, 2.0, 4.0, and 6.0 molecular equivalents (approximately) of glucose. With increasing proportions of glucose there were increasing rates of acetoacetate consumption, the consumption with 2 or more equivalents being nearly complete[1] in 2 hours. Taking the results after 4 hours as a basis of comparison, and calling the consumption with 2 molecular equivalents of glucose 100 per cent, we have

				Acetoacetate consumed. *mMols*		*per cent*
With 2	mols	glucose	equivalent	= 16.8	=	100
" 1	"	"	"	= 16.9	=	±100
" 0.5	"	"	"	= 12.0	=	71
" 0.25	"	"	"	= 7.8	=	46
" 0	"	"	"	= 1.6	=	10

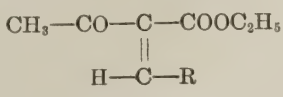
From this it would appear that over 0.5, but less than 1.0, glucose equivalent is required for the ketolytic oxidation of acetoacetate. With 0.25 molecular equivalent of glucose less than half of the acetoacetate was consumed. We shall consider later the ketolytic ratio of sugar to acetoacetate. The above data are given at this point only to emphasize the quantitative relationship between them in the reaction.

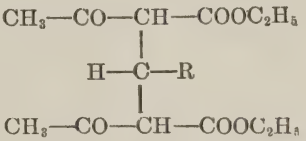
It would seem that the behavior illustrated by the data shown above can be most easily explained as the result of a chemical reaction between acetoacetate and *some intermediate* in the oxidation of glucose. The intermediate, which has doubtless only a fleeting existence, must have such an affinity for acetoacetic acid as to permit the condensation to outrun the rate of its oxidation. To establish the identity of the reactive ketolytic derivative and the nature of its condensation product, and to determine the further fate of the latter on oxidation would complete the story of the "ketolytic reaction." It proves, however, to be a very difficult and elusive problem to solve; and another explanation seems more likely.

We may first consider the type of reaction involved in the condensation of the ketolytic substance with acetoacetate. It was pointed out by one of us when the reaction was first reported (7)

[1] The residual acetoacetate is chiefly due to a part being spontaneously decomposed into acetone (and CO_2), which in the determination is calculated as acetoacetate.

that it was probably a special case of the Claisen or Knövenagel type of condensation (8), by which a great variety of aldehydes and ketones are known to react with acetoacetic ester. Knövenagel (8) showed that either 1 or 2 molecules of ester, depending upon temperature, may be condensed with a molecule of aldehyde, yielding by the loss of water alkylidine mono- or bis-acetoacetic esters of the following types.





Except at very low temperatures, the bis type of condensation usually occurs. Although some points remain uncertain, our work indicates that the ketolytic action of glucose and other sugars is due to a condensation of the second type, the essential difference being that the salt of acetoacetic acid takes the place of the esters and an oxidation product of the sugar (or the sugar itself) replacing the aldehyde in the Knövenagel reaction.

The possibility has been earlier recognized that this type of condensation might have a rôle in biochemical phenomena. In 1904 Schryver (9) wrote:

> "When it is remembered that aceto-acetic acid is secreted together with sugar in cases of diabetes mellitus . . . . the possibility is immediately suggested that a reaction of the Knoevenagel type is possible in the organism, especially as the chemical probability exists that various aldehydes are formed, at any rate as intermediary products, by the oxidation of glucose, which should be capable of condensing with aceto-acetic acid."

Schryver's statement is an accurate prediction of what we term the ketolytic reaction. But, having in mind the influence of piperidine and other bases as condensing agents, demonstrated in Knövenagel's work, Schryver's attention was directed to the possible action of nitrogenous bases of the tissues as catalysts in this type of reaction. His efforts to demonstrate such action (with acetoacetic ester) were unsuccessful. Dakin (10) also has referred to the apparent analogy between this type of reaction and biochemical syntheses, but no direct application to biochemical phenomena appears to have been made.

The approach to the question of the identity of the ketolytic derivative of glucose or other hexoses appears to traverse the difficult field of the complex transformations of sugars by alkali, studied especially by de Bruyn and van Ekenstein, by Nef, and by others (11), as well as the equally difficult subject of the path of oxidation of the "dissociation" products formed by alkali. It will be noted that the curves of the rate of glucose oxidation given in Charts 2 and 3 have a slight S-shape, showing an initial lag in the oxidation. This indicates a fact, noted in our first paper (1), and which can be demonstrated in other ways, that it is *not* ordinary glucose which is oxidized, *but a derivative resulting from the action of alkali.* In the presence of oxygen as well as alkali, an *intermediate product*, somewhere along the trail of enolizations and dissociations of the sugar caused by alkali alone (not the end-products), is oxidized; and the ketolytic derivative is again presumably an *intermediate* in the path of oxidation,—it is not the end-product. These very complex reactions with the hexoses make their ketolytic reactions too difficult to decipher, and we have accordingly turned to the simpler aldose, glycol aldehyde. This sugar exhibits the typical ketolytic reaction exactly comparable to the hexoses; and because of its smaller molecule and simpler structure it is somewhat less difficult to follow its transformations under the action of alkali and on oxidation. In a later paper we shall attempt an analysis of the ketolytic reaction with hexoses, in the light of the results here presented.

Glycol Aldehyde.

Glycol aldehyde[2] "consumes" acetoacetic acid under two conditions. (1) Like glucose and other sugars, when undergoing oxidation by H_2O_2 in alkaline solution, it rapidly accomplishes the oxidation also of acetoacetic acid. This we shall refer to as the "oxidative ketolytic condensation." (2) Glycol aldehyde condenses with acetoacetic acid *also without oxidation* and this type of reaction we may call the "direct ketolytic condensation." Other simple aldehydes exhibit a similar behavior, some instances

[2] We are indebted to Dr. I. Greenwald for his kindness in giving us a sample of pure crystalline glycol aldehyde prepared by him by the Fenton (12) method.

of which will be recorded in a separate paper. This direct reaction between aldehydes and acetoacetic acid, a simple Knövenagel condensation of the reacting substances, although in some re-

TABLE IV.

Rate of Oxidation of Glycol Aldehyde and Acetoacetate, Separately and Together, by H_2O_2 in 0.5 N KOH at 38°C.

Experiment No.	131	134	135	132	133
	mMols	*mMols*	*mMols*	*mMols*	*mMols*
Added per liter.					
Glycol aldehyde	18.6	18.6	0	18.6	0
K acetoacetate	0	81.4	81.4	40.7	40.7
H_2O_2	200	400	400	200	200
	cc. N	*cc. N*	*cc. N*	*cc. N*	*cc. N*
Acid formed.					
2 min.	27.0	38.9			
1 hr.	38.6	157.0	30.4	139.6	11.5
2 hrs.	38.6	189.0	50.6	174.4	18.1
3 "	38.1	202.4	74.9	190.1	18.8
22 "				203.3	109.8
		mMols	*mMols*	*mMols*	*mMols*
Acetoacetate consumed.					
2 min.		17.1	0		0.1
1 hr.		36.7	6.6	32.4	1.5
2 hrs.		39.7	8.3	37.7	3.0
3 "		40.6	13.6	39.6	3.5
3.5 "		42.1			
22 "				40.3	20.1
		mMols	*mMols*		
CO_2 formed.					
3 min.		4.3	2.9		
1 hr.		37.5	12.1		
2 hrs.		49.0	18.8		
3 "		55.6	26.5		

spects apparently different, may be actually the basis of the typical oxidative ketolysis.

1. "Oxidative Condensation" with Glycol Aldehyde.

When glycol aldehyde is dissolved in an excess of 0.1 to normal caustic alkali the solution gradually darkens and the sugar is

converted into a complex mixture of polymerization and decomposition products among which are "saccharinic acids." These *end-products* are in large part resistant to later oxidation by peroxide. If, however, H_2O_2 be added at the start the sugar is *much more rapidly* oxidized to formic acid. At 37°C. in 0.5 N KOH the oxidation is complete in less than 1 hour and *almost exactly 2 mols of formic acid are formed from each mol of the sugar.* The *end*-product, formic acid, is *not ketolytic.* But if to another equal portion of glycol aldehyde there be added more than 2 molecular equivalents of acetoacetate and the mixture be oxidized by H_2O_2 in 0.5 N KOH under the same conditions, the acetoacetate disappears at a rate closely corresponding to the rate of aldehyde oxidation, with very greatly increased acid production. In the absence of glycol aldehyde the oxidation of acetoacetate is quite slow.

These relations are shown by the data given in Table IV and illustrated in Chart 4, giving by curves the rates of acid production from the oxidation of glycol aldehyde alone and from the oxidation of glycol aldehyde + acetoacetate, and the rates of acetoacetate consumption in the presence and absence of glycol aldehyde. It is evident from the curves that the rate of consumption of acetoacetate is enormously increased by glycol aldehyde. The oxidation of glycol aldehyde to formic acid is complete in less than an hour, and only before (or during) its oxidation to formic acid can it react with, and thus "consume," acetoacetate. There is, to be sure, a continued consumption of acetoacetate beyond the 1st hour, but close inspection of the data and the curves shows that this is due only to the continued direct oxidation of the residual excess acetoacetate. The complete oxidation of the product of the condensation appears to be somewhat slower than that of glycol aldehyde as indicated by the continued acid production in the ketolytic experiments after 1 hour, at which time the acid formed from the aldehyde alone is maximum.

Comparison of the rate of CO_2 production in the presence of glycol aldehyde (No. 134, Table IV, and CO_2 curves in Chart 4) shows that by the end of the 1st hour, that is, during the period of condensation, the CO_2 formed by decarboxylation of the condensation product very nearly equals the acetoacetate consumed. In other words, *1 mol of CO_2 is split off from each mol of acetoacetate*

soon after its condensation. The later continued CO_2 production parallels the rate from acetoacetate alone, and is evidently due

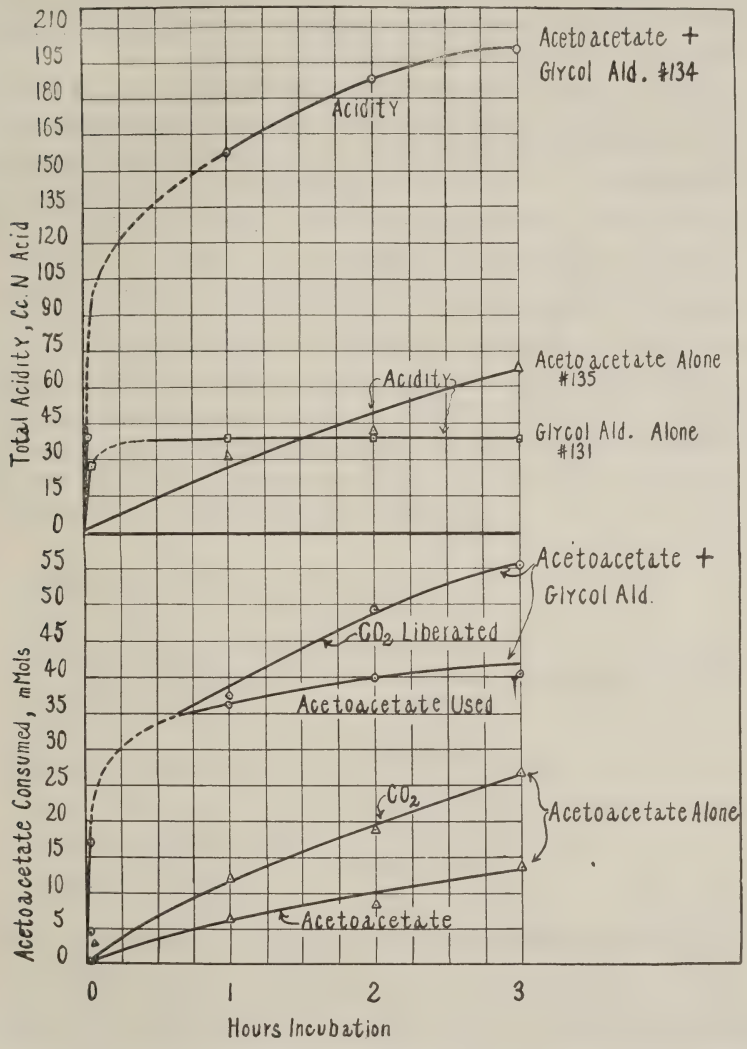


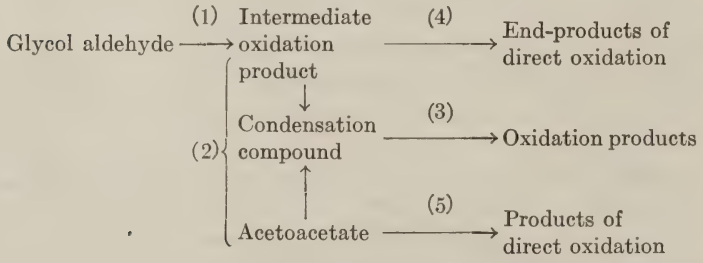
Chart 4. Glycol aldehyde + potassium acetoacetate in 0.5 N KOH + H_2O_2 at 37.5°C. Rate of oxidation of glycol aldehyde compared with acetoacetate consumption, CO_2, and acidity with and without the aldehyde.

merely to direct oxidation of residual acetoacetate. The CO_2 formed in the absence of glycol aldehyde (No. 135) corresponds to approximately 2 mols from each mol of acetoacetate, and is formed by the slow direct oxidation of the keto acid, which yields, besides the 2 mols of CO_2, 1 mol of acetic acid.

It will be noted that the total acetoacetate consumed is substantially the same in Nos. 134 and 132, with equal amounts of glycol aldehyde, but in the latter only one-half the concentration of acetoacetate. The keto acid consumption is, therefore, *limited by the amount of aldose oxidized*, being approximately *2 mols* of acetoacetate for each mol of aldose.

It would appear that the most probable explanation of the great increase in the rate at which acetoacetate is oxidized in the presence of glycol aldehyde is that it is due to a Knövenagel type of condensation between an *intermediate* oxidation product of the aldehyde and 2 mols of the keto acid, the condensation compound being then oxidized at a rate which is slightly less than that of the uncombined aldehyde and much more rapidly than the uncombined acetoacetate. According to this explanation, the reactions may be outlined as follows:

Scheme I.



Of these reactions, those which lead to the formation of the ketolytic derivative (1), its condensation with acetoacetate to form the condensation compound (2), and the oxidation of the latter (3), are the ones with which we are especially concerned. What is the ketolytic intermediate formed in reaction (1)?

In its oxidation to formic acid by alkaline H_2O_2 glycol aldehyde is believed to pass through glyoxal (Heimrod and Levene (13)).

This conclusion is unavoidable, because neither of the two other possible derivatives, glycollic and glyoxylic acids, yield formic acid quantitatively under the same conditions, and both are, therefore, excluded as intermediates in the oxidation. Glyoxal, however, *is* quantitatively oxidized to formic acid, and, under certain conditions, *condenses very rapidly with acetoacetic acid.*

These facts point to glyoxal as the ketolytic derivative of glycol aldehyde in its oxidative ketolysis. Another fact, however, urges caution in reaching this conclusion, if it does not exclude it. *When treated under apparently the identical conditions which are optimum for oxidative ketolysis with glycol aldehyde, glyoxal has relatively slight action on acetoacetate.* That is, in the presence of alkali and peroxide, glyoxal is only slightly ketolytic. The reasons for this failure are evident from the peculiar properties of glyoxal described below. In spite of the fact that under other conditions glyoxal *does* exhibit very marked and rapid reactivity toward acetoacetate, we are unable to explain away the objection above stated.

Behavior of Glyoxal.

When solutions of glyoxal[3] are mixed with approximately neutral or slightly alkaline solutions of acetoacetate, the latter gradually disappears, with practically no change in the reaction. Carbonic acid is formed at a parallel but slightly slower rate; the alkali, combined at the start with acetoacetate, being left as bicarbonate. The rate of the acetoacetate consumption depends upon the reaction of the solution. In neutral or faintly alkaline solution it is slow but continues for days, and the total consumption is large. With increasing alkalinity the rate rapidly increases, but the reaction soon stops and the total acetoacetate consumed may be much less than at lower alkalinity. The optimum reaction for both fairly rapid and high condensation with acetoacetate is about that

[3] Glyoxal was prepared according to the method of Ljubawin (14) by the oxidation of paraldehyde with nitric acid. After the reaction had come to completion the reaction mixture was repeatedly distilled *in vacuo* at 50°C. This removed paraldehyde, acetic acid, glyoxylic acid (?), and most of the mineral acid. The clear, colorless syrup of polyglyoxal was then diluted to about molar strength. These solutions, which contained free mineral acid (0.1 to 0.2 N), were used in all our experiments.

TABLE V.—*Effect of Reaction (pH) and Concentration on th*

Added per liter. Glyoxal..... Acetoacetate.....	Experiment B_1. $NaHCO_3$ at 25°C. ± 2 per cent. 90 mMols. 140 "			Experiment 207. Na_2HPO_4 at 37.5°C. 50 cc. M per l. 62 mMols. 108 "			Experiment B. Na_2CO_3 at 25°C. ± 3 per cent. 90 mMols. 140 "			Experiment D. $Na_3PO_4 + Na_2HPO_4$ at 25°C. 90 mMols. 140 "		
	Time.	Acetoacetate consumed.	Theoretical maximum.	Time.	Acetoacetate consumed.	Theoretical maximum.	Time.	Acetoacetate consumed.	Theoretical maximum.	Time.	Acetoacetate consumed.	Theoretical maximum.
	min.	*mMols*	*per cent*	*min.*	*mMols*	*per cent*	*min.*	*mMols*	*per cent*	*min.*	*mMols*	*per cent*
	5.5	4.0	2.2	5	26.4	21.3	5.5	48.2	26.8	5.5	6.0	3.3
	29.5	20.9	11.6	30	42.8	34.5	32.0	86.4	48.0	27.5	22.8	12.7
	76.5	31.5	17.5	60	47.4	38.4	79.0	94.5	52.5	74.0	33.0	18.3
	124	37.8	21.0	120	48.9	39.4	127.0	95.1	52.8	122.0	42.1	23.4
	210	52.1	28.9				212.0	95.8	53.2	209	51.9	28.8
	426	70.9	39.4				427.0	93.8	52.0	424	70.1	38.9
	1,356	97.4	54.1				1,357	92.8	51.6	1,354	93.2	51.8

of mixtures of $NaHCO_3 + Na_2CO_3$, or a pH range of 10 to 12. In the presence of an excess of alkali hydroxide such condensation as occurs takes place almost instantly, but the total is small. With an excess of 0.2 to 0.5 N KOH and H_2O_2, which condition is optimum for the ketolytic action of glycol aldehyde and other sugars, the acetoacetate consumption with glyoxal is almost *negligible*. The data given in Table V and Charts 5 and 6 show the rate and extent of acetoacetate consumption under different circumstances.

Its slowness to react at very low alkalinity and its failure to react at high alkalinity, with and without peroxide, are explained by the following behavior. Ordinary solutions of glyoxal consist chiefly of a stable polymeric form in equilibrium with small amounts of the very reactive monomolecular form (15). In the presence of an excess of alkali it is very rapidly converted into glycollic acid (16), the rate of the transformation being determined by the alkali concentration. The effect of the alkali is presumably due to its favoring the enolization of the monomolecular form. The rate of its conversion to glycollic acid at different

ate of Condensation of Acetoacetate with Glyoxal.

Experiment A. Na_2CO_3 at 25°C. 0.2 saturation. 90 mMols. 350 "			Experiment C. Na_2CO_3 at 25°C. 0.2 saturation. 22.5 mMols. 140 "			Experiment B_2. Na_2CO_3 at 25°C. Saturated solution. 180 mMols. 548 "			Experiment 2. KOH at 25°C. 0.4 N 40 mMols. 94 "			Experiment 420. KOH at 27-30°C. 0.2 N 27.0 mMols. 61.6 "			Experiment 421. KOH + H_2O_2 at 27-30°C. 0.2 N KOH 27.0 mMols. 61.6 "		
Time.	Acetoacetate consumed.	Theoretical maximum.	Time.	Acetoacetate consumed.	Theoretical maximum.	Time.	Acetoacetate consumed.	Theoretical maximum.	Time.	Acetoacetate consumed.	Theoretical maximum.	Time.	Acetoacetate consumed.	Theoretical maximum.	Time.	Acetoacetate consumed.	Theoretical maximum.
in.	*mMols*	*per cent*	*min.*	*mMols*	*per cent*	*min.*	*mMols*	*per cent*	*min.*	*mMols*	*per cent*	*min.*	*mMols*	*per cent*	*min.*	*mMols*	*per cent*
20	139	77.5	20	30	67.0	3	176	49	6	11.6	14.5	0.62	6.60	12.2	0.45	1.70	3.1
40	146	81	40	34.5	73	7	228	63	23	11.0	13.8	1.23	6.45	11.9	0.98	0.40	0.7
60	148	82	60	34.8	77	20	288	80	270	9.9	12.4				2.45	0.75	1.4
130	146	81	130	35.5	79	32	286	80							10.0	2.70	5.0

alkalinities is illustrated in Chart 7 and Table VI. It will be seen that the conversion does not occur (or only very slowly) in a solution buffered by $NaHCO_3 + CO_2$, having pH about 7.5; is measurable but slow in $NaHCO_3 + Na_2CO_3$, pH $\pm$ 11; is much faster in Na_2CO_3, pH probably about 12 (?); and is almost instantaneous in an excess of NaOH. Free acidity of glyoxal solutions may be accurately titrated with phenolphthalein as indicator (pH 10) but the glyoxal is quantitatively converted to glycollic acid on warming with a moderate excess of 0.1 N NaOH. The reason, therefore, that glyoxal shows little or no ketolytic activity at high alkalinity is that its conversion into (non-ketolytic) glycollic acid is so rapid at this reaction as to exceed the condensation.

At lower alkalinity within the zone pH 7 to 10, where the glyoxal is not being removed by glycollic acid formation, the rate of condensation with acetoacetate increases with rise of alkalinity, as shown in the curves of Charts 5 and 6. This effect of the alkali may be due either to shifting the equilibrium of polyglyoxal $\leftrightharpoons$ monoglyoxal to the right through enolization and thereby increas-

ing the concentration of the reactive form, or the alkali may catalyze the condensation of glyoxal and acetoacetate, perhaps by removal of the product by splitting off CO_2.

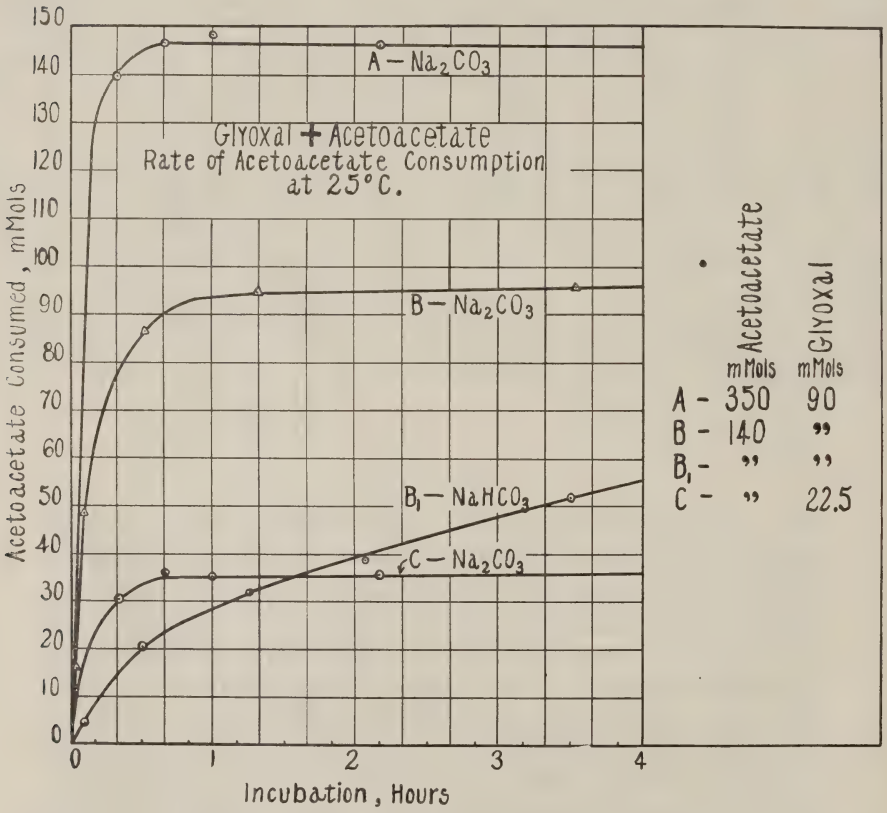


Chart 5. Rate of condensation of glyoxal with sodium acetoacetate at 23–25°C. The rate is very greatly altered by the reaction (compare *B* and B_1) and by the relative, as well as the absolute, concentrations of the reacting substances (compare *A*, *B*, and *C*). The effect of concentration and reaction is perhaps more clearly illustrated by Chart 6.

The reason why glyoxal is not ketolytic in the presence of peroxide as well as of alkali is a different one—because of its great ease of oxidation to formic acid. At moderately high acidity (above pH about 4) it is resistant to H_2O_2, while at very slight alkalinity it is exceedingly rapidly oxidized,—so rapidly that the oxidation outruns

the conversion to glycollic acid, in the presence of peroxide, even in 0.5 N KOH. (Glyoxal is quantitatively oxidized to formic acid on being added with stirring to an excess of H_2O_2 and KOH.)

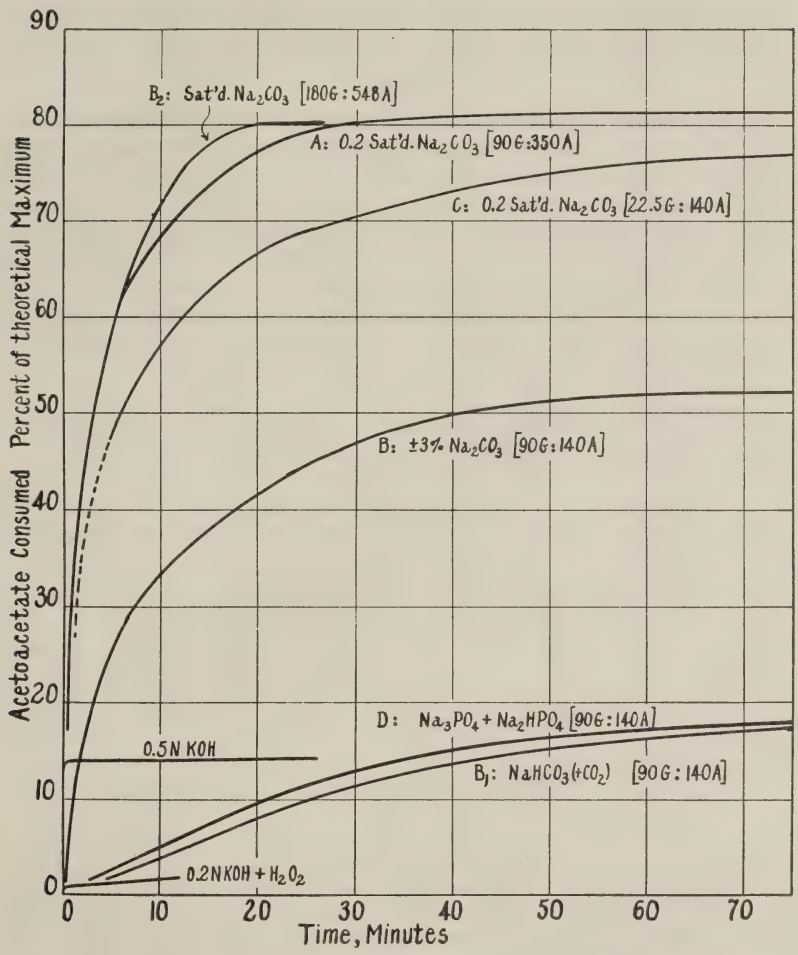


CHART 6. Rate of condensation of glyoxal with sodium acetoacetate at 23–25°C.

The striking effect of the reaction upon the rate of oxidation is demonstrated by the following simple experiment. If a solution of glyoxal, to which has been added phenolphthalein and methyl orange, be titrated with dilute alkali, it is found that when the

pink color of phenolphthalein is reached it is permanent, thus indicating that no acid (glycollic) is produced at that reaction. If, now, there be added H_2O_2 (previously made faintly alkaline to phenolphthalein), the pink color is instantly abolished, but the solution remains alkaline to methyl orange. On further addition

TABLE VI.

Rate of Conversion of Glyoxal to Glycollic Acid at Different Reactions at 25°C.

Glyoxal (25 cc. of M solution, containing 4.27 cc. of N free acid) was added to $NaHCO_3$, Na_2CO_3, and NaOH solutions (small portions of which were neutralized by the free acid of the glyoxal solution). The mixtures were at once diluted to 500 cc., and 50 cc. aliquots were measured at the time stated into 50 cc. of 0.1 N HCl (an excess). The acid solutions were boiled, cooled, and titrated to phenolphthalein. The acid formed from the glyoxal, calculated for the total volume, is expressed in percentage of the total; that is, the maximum formed on converting all the glyoxal into glycollic acid by boiling with a slight excess of NaOH.

25 mMols in $NaHCO_3$(+ CO_2). ±0.05M pH ± 7.5 (initial).		25 mMols in Na_2CO_3(+ $NaHCO_3$). 0.025M pH ± 11 (initial).		25 mMols in 0.05 N NaOH. pH ± 12.5 (initial).		18.2 mMols in 4 per cent Na_2CO_3* at 38°C.	
Time.	Converted to glycollic acid.	Time.	Converted to glycollic acid.	Time.	Converted to glycollic acid.	Time.	Converted to glycollic acid.
min.	*per cent*		*per cent*	*min.*	*per cent*	*min.*	*per cent*
12	0	2 min.	0.3	1	100	1.5	8.6
30	0	6 "	1.9	2	100	3.0	15.9
223	0	13 "	4.3	4	100	4.75	19.7
		30 "	8.7			10	39.4
		60 "	12.9			15	47.0
		105 "	17.3			30	62.8
		25 hrs.	40.5			60	77.8
						90	82.0
		25 "	pH ± 8.4			300	89.3

* The data in this experiment were obtained by determining the *residual* glyoxal. Aliquots were run into a solution of alkali and H_2O_2. Under these conditions glyoxal yields formic acid quantitatively; glycollic acid is not oxidized.

of alkali the pink of phenolphthalein does not appear until most of the glyoxal has been oxidized; the oxidation runs so fast that the continuous addition of alkali does not overtake the acid until the glyoxal is almost all gone. The same experiment may be done

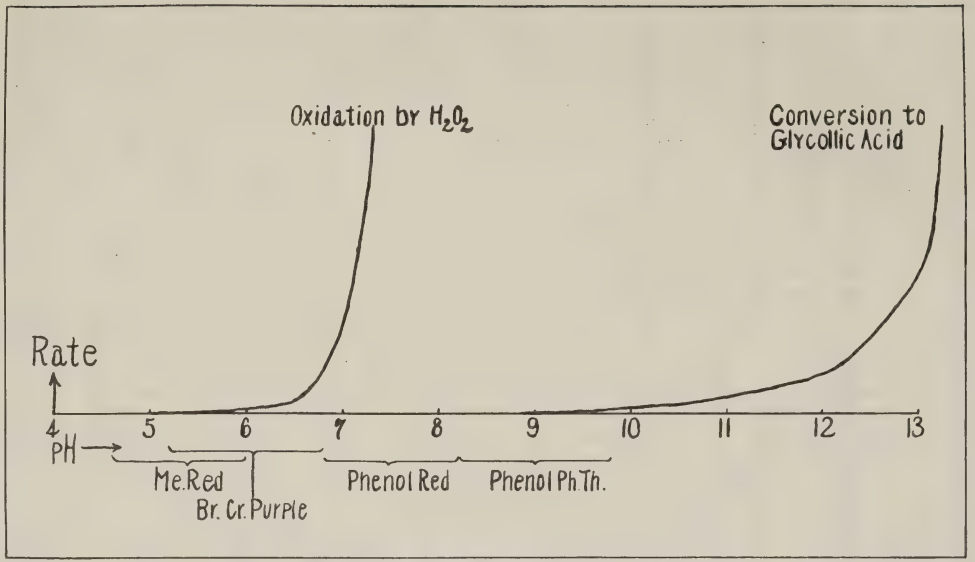


CHART 7. Diagram to represent the effect of reaction (pH) upon the oxidation of glyoxal by H_2O_2, and its conversion to glycollic acid. These data are only rough approximations and are based upon the results shown in Table VI.

using methyl red and phenol red, with the result that the oxidation proceeds very rapidly at pH 7 to 8, and takes place, though slowly, at pH 5. *At a given reaction the oxidation takes place more rapidly than the condensation with acetoacetate, and this explains why glyoxal*

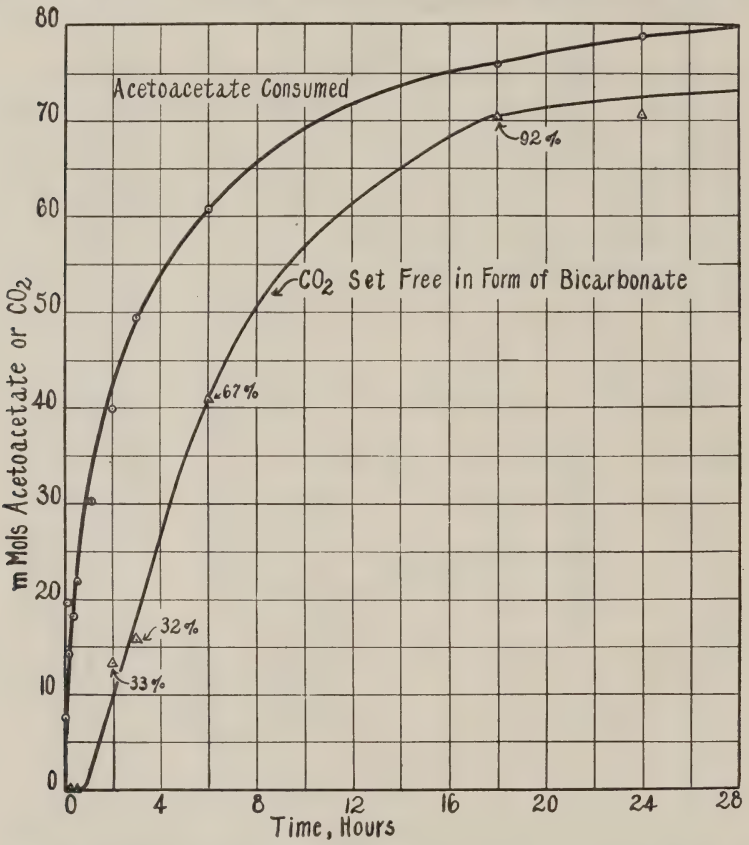


Chart 8. Experiment 407. 50 mMols of glyoxal + 101.5 mMols of potassium acetoacetate. Showing the relation between acetoacetate consumption and liberation of CO_2 at pH = 7.5 and 37–38°C.

is not ketolytic in the presence of peroxide. The above facts are indicated diagrammatically in Chart 7.

The fact that in quite acid solution glyoxal resists oxidation, might be interpreted to mean either that only monoglyoxal is oxidizable by H_2O_2 and that in strongly acid solutions this form does not exist, or that the reaction determines the activity of the

peroxide. The latter appears the more probable because there is other evidence to indicate that reactive glyoxal, presumably the monomolecular form, is present even in strongly acid solutions. That evidence is the fact that the addition of acidified solutions of *p*-nitrophenylhydrazine at once precipitates the red glyoxal dihydrazone. The reaction of the solution, therefore, probably imposes no hindrance to the rapid depolymerization when the equilibrium is disturbed; and active glyoxal potentially exists in any solution of the polymeric form.

If the last statement be true, the slow rate of oxidation in acid solution and the slow rate of condensation with acetoacetate at low alkalinity cannot be due to the absence or low concentration of the monomolecular form, and must rather be ascribed to some factor concerned either with increasing the reactivity of glyoxal (or acetoacetate), or in accelerating the reactions by removing the products. In the case of the condensation with acetoacetate, it seems probable that the alkali promotes the reaction by removing CO_2 from the intermediary condensation product. This is indicated by the fact already noted, and shown in Chart 8, that CO_2 appears in the reaction at approximately a parallel, although slower, rate than that of acetoacetate consumption.

TABLE VII.

Rate of Acetoacetate Consumption and Rate of Liberation of CO_2 at pH = 7.5 and 37–38°C.

Experiment 407. 50 mMols glyoxal and 101.5 mMols acetoacetate.

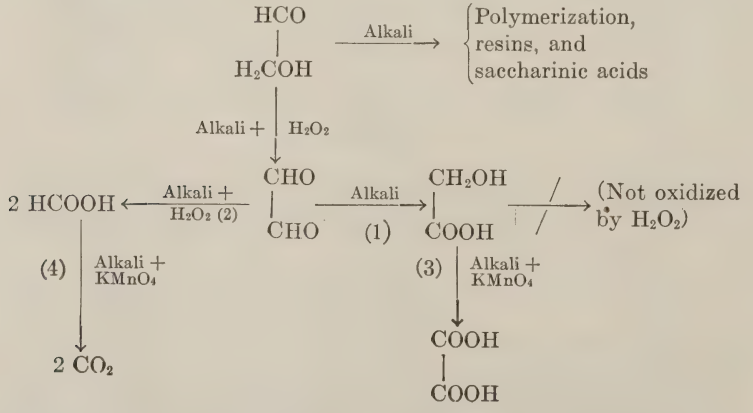
Time.	Acetoacetate consumed.	CO_2 liberated.*
	mMols	*mMols*
2 min.	7.6	0
6 "	19.7	0
12 "	14.3	0
22 "	18.2	0
33 "	21.8	0
68 "	30.3	
2 hrs.	39.9	13.3
3 "	49.4	15.8
6 "	60.8	40.8
18 "	76.1	70.3
24 "	78.9	70.6

* CO_2 was determined as follows: An aliquot was run into 100 cc. of a cold, slightly alkaline solution of $BaCl_2$. After shaking vigorously and allowing to stand ½ to 1 minute, the precipitate of $BaCO_3$ was filtered, washed, and titrated with acid. The results shown above have been corrected by blanks.

Another interesting consequence of the behavior of glyoxal may be noted here. Depending upon the order of treatment, alkali, H_2O_2, and $KMnO_4$ convert it practically quantitatively, in one case into oxalic acid, and in the other into CO_2. If an excess of alkali be added first, glycollic acid is formed which is not affected by subsequent addition of H_2O_2, but is promptly oxidized to oxalic acid by $KMnO_4$ (in alkaline solution). But if the H_2O_2 be first mixed with the alkali, and glyoxal be then added, it is oxidized to formic acid which on later treatment with $KMnO_4$ is converted into CO_2. The reactions above described are illustrated by the following data and are summarized in the diagram.

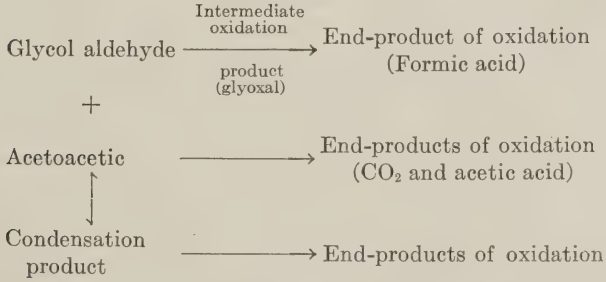
Glyoxal treated with:	From 10 cc. of glyoxal solution.
(1) 0.5 N KOH	18.37 cc. N KOH neutralized.
(2) 0.5 " " + H_2O_2	39.38 " " " "
	38.43 " " volatile acids.
	38.15 mMols formic acid.
(3) 0.5 " " ; followed by $KMnO_4$	19.45 " oxalic "
(4) 0.5 " " + H_2O_2; followed by $KMnO_4$	38.42 " CO_2.

Reactions (1) and (2) have been used by us to determine the concentration of glyoxal solutions. More concordant results are obtained when the reactions are carried out in more dilute alkali and with smaller amounts of glyoxal.



The behavior of glyoxal above described appears to explain its failure to react with acetoacetate under the conditions of the oxidative ketolysis. And in view of this explanation, we are unable to formulate any satisfactory hypothesis by which glyoxal can be retained as the ketolytic derivative in the oxidative reaction. We are thus led to the paradoxical situation, that the *only* oxidative derivative of glycol aldehyde which might be the ketolytic derivative cannot be the ketolytic derivative.[4] Apparently the only escape from this dilemma is to turn to glycol aldehyde itself. The only possibility that appears to be left is that *the very rapid oxidative ketolysis is merely the direct (non-oxidative) condensation of glycol aldehyde itself with acetoacetate, greatly accelerated by the removal of the product by oxidation.* As stated on an earlier page glycol aldehyde condenses with acetoacetate also in the absence of peroxide, and this direct condensation may be the start of the oxidative ketolytic reaction. According to this idea we may outline the reactions as follows.

Scheme II.



The difference between Schemes I and II is the point that in Scheme I the condensation of acetoacetate takes place with a product of the oxidation of the sugar, while in Scheme II it reacts with the aldose *before* oxidation. The latter conception appears to be possible only on the assumption that the condensation is a reversible reaction, which occurs only to a slight extent unless the

[4] Glyoxals may perhaps be ketolytic in the animal body, provided the rate of their destruction by oxidation or by glyoxalase does not too greatly exceed the rate of condensation with acetoacetate.

product of the condensation is removed as formed. There are certain difficulties in this view also, but so far as we have been able to see these are the only possibilities.

Non-Oxidative Ketolytic Condensation of Glycol Aldehyde.

When mixed in very slightly alkaline solution, glycol aldehyde reacts with acetoacetate, though the consumption is considerably less than during oxidation. In strongly alkaline solution the consumption is very small.

Experiment 257.—Glycol aldehyde (0.3 gm., equivalent by analysis to 4.65 mMols) was dissolved in water, 10 cc. of 1.17 M potassium acetoacetate were added, and the solution was diluted to 200 cc. The concentrations correspond to 23.3 mMols of aldehyde and 58.4 mMols of acetoacetate per liter. The reaction of the mixture was about pH 8. After 20 hours at 37°C. the solution contained 35.4 mMols of acetoacetate per liter, or 23 mMols had been consumed, and had gained 24.9 mMols of CO_2. On adding a known excess of 0.1 N NaOH and $BaCl_2$ to an aliquot and titrating, the acid formed (over a blank without glycol aldehyde) was found to be 31.1 cc. of N per liter. Adding the acid neutralized by the base available from the consumed acetoacetate (23 mMols), the total acid is 54.1 cc. of N, 49.8 cc. of which (24.9 × 2) are due to CO_2 (titrated as dibasic acid after precipitation as $BaCO_3$).

The total consumption of acetoacetate is in this case almost exactly 1 mMol for each mMol of aldose present, while in the oxidative ketolysis about twice that amount is destroyed.

Experiment 101.—Glycol aldehyde (0.24 gm. = 3.7 mMols) was dissolved, added to 9.3 mMols of acetoacetate and KOH (final concentration 0.5 N), and diluted to 200 cc. After 10 minutes, distillation of an aliquot from acid solution showed that 0.28 mMols had been consumed. At the end of 20 hours only 0.38 mMols had disappeared. In the presence of strong alkali, therefore, the amount of ketolysis is very small. A parallel experiment, with the same quantities and containing also H_2O_2, showed 3.6 mMols of acetoacetate consumed in 1 hour and 6.8 mMols after 20 hours.

Products of the Ketolytic Reactions.

Having been unable so far to isolate the products of the condensation, because of their instability and destruction during the reaction, we have attempted to secure evidence concerning them from a quantitative determination of their oxidation products. In the oxidative ketolytic reactions the end-products are, of course, those

from the oxidation of all the reacting substances. Similar oxidation products are obtained from the direct (non-oxidative) condensation, by oxidizing the mixture by alkaline H_2O_2 *after* the direct condensation has taken place. In this way we may compare the products of the oxidative reaction with, on the one hand, the oxidation products of the direct condensation with glycol aldehyde, and on the other, with the corresponding oxidation products from the condensation with glyoxal. The products of whichever pathway (via glyoxal or via direct glycol aldehyde condensation) agree with the products of the oxidative reaction will presumably indicate its course.

Under each of these conditions the products found obviously represent (1) portions of the reacting substances (glycol aldehyde or glyoxal and acetoacetate), which have been directly oxidized before undergoing condensation, and (2) the condensation compound. The problem is, to learn the products of the latter.

We shall consider first the products of the oxidative reaction with glycol aldehyde.

The direct oxidation of acetoacetate gives under these conditions, 1 mol of acetic acid and 2 mols of CO_2; and both glycol aldehyde and glyoxal yield 2 mols of formic acid. Besides these products there is found from the ketolytic reactions with glycol aldehyde only one other acid, which is non-volatile, ether-soluble, has one acid equivalent for each 2 carbon atoms, and, on further oxidation by alkaline $KMnO_4$, is nearly quantitatively converted into oxalic acid. Although this acid has not been otherwise identified, the above facts indicate that it is glycollic acid. Since this substance is not formed by the oxidation of either glycol aldehyde or acetoacetate under the same conditions it must be derived from the oxidation of the condensation product. On this assumption we may take the amount of oxalic acid found after $KMnO_4$ oxidation of the products of the ketolytic reaction (after removal of residual acetoacetate and acetone) as a measure of the glycollic acid, and therefore of the condensation compound, and calculate the other products of its oxidation.

A summary of the analytical results of a number of these experiments is given in Table XI.

Analytical Methods.

Carbon Dioxide.—An aliquot was slightly acidified, and the CO_2 was rapidly aerated at room temperature through 30 cc. of 0.1 N NaOH contained in a bead tower. After 15 minutes aeration the tower was washed free of alkali by 150 to 200 cc. of CO_2-free water. The carbonate was precipitated by adding $BaCl_2$ and the residual alkali was titrated by 0.1 N HCl to the phenolphthalein end-point. The results are accurate to within less than 0.5 per cent when properly corrected by blanks.

Acetoacetic Acid.—An aliquot (yielding 20 to 40 mg. of acetone) was distilled from strongly acidified solution and redistilled after adding Na_2O_2. Acetone was collected in 200 to 300 cc. ice-cold water and determined iodometrically by the well known Messinger method.

Total Acidity.—This was determined by titration of the residual base after adding an excess of $BaCl_2$, the titration being continued until the solution was *permanently* colorless to phenolphthalein. The difference between the residual base found by titration and the base added (determined in a blank) is due to the acid formed. H_2CO_3 is titrated as a dibasic acid under these conditions. To the result by titration there must be added the acid neutralized by base made available from consumed acetoacetate.

Volatile Acids.—H_2O_2 of the original solution was decomposed by MnO_2, and the solution was filtered. 200 to 300 cc. of the *clear* filtrate were acidified by 5 N H_2SO_4 and were slowly distilled with steam until 2 liters of distillate were collected. 500 cc. of the distillate were rapidly aerated 2 to 3 minutes with CO_2-free air and titrated with 0.1 N NaOH to the phenolphthalein end-point. Blank distillations were simultaneously made with slightly acidified distilled water and with the acetoacetate solution. The latter always contained small quantities of acetic acid.

Formic Acid.—An aliquot of the steam distillate (containing acid equivalent to 30 to 60 cc. of 0.1 N NaOH) was made slightly alkaline and gently boiled 10 to 15 minutes to remove acetone. 50 cc. of formic acid reagent (50 gm. of $HgCl_2$ + 27.5 gm. of $NaC_2H_3O_2$ per liter) were added after making slightly acid by glacial acetic acid. The solution was then digested 5 hours in the boiling water bath. The precipitated calomel was transferred to a tared

crucible, washed with distilled water, two to three times with 5 per cent HCl, and finally again washed with distilled water, dried at 120°C., and weighed. Blank determinations were always made. Factor: mg. HgCl × 0.0977 = mg. of formic acid.

Acetic Acid.—This was always determined by difference. The total volatile acidity − formic acid = "acetic acid."

Oxidation of Non-Volatile Residue by $KMnO_4$; "Glycollic Acid."—The residue from the steam distillation was transferred to a 500 cc. volumetric flask which contained 25 cc. of 2 N KOH. An excess of 0.5 M $KMnO_4$ was then slowly added. The solution was warmed on the electric hot-plate and finally brought to boiling. It was cooled; excess $KMnO_4$ was destroyed by adding 3 per cent U. S. P. H_2O_2; and the solution was brought to volume and filtered. An aliquot of the *clear* filtrate was acidified with glacial acetic acid till acid to phenol red. The solution was heated to boiling and an excess of M calcium acetate solution was added. It was then allowed to digest overnight at room temperature. The precipitate of calcium oxalate was filtered on a Gooch crucible and titrated by $KMnO_4$.

We may take Experiment 136, an oxidative reaction with glyco aldehyde, to illustrate the procedure and the method of calculating the results.

Experiment 136.—18.6 mMols of glycol aldehyde and 40.7 mMols of acetoacetate were oxidized by 200 mMols of H_2O_2 in a liter of 0.5 N KOH. On analysis of the solution after 18 hours 0.6 mMol of acetoacetate was recovered (and removed) as acetone, leaving 40.1 mMols which had been "consumed" or oxidized. The products which would have been formed by the direct oxidation of the sugar (18.6 mMols) and of the keto acid separately and the products actually found are given in Table VIII.

The total carbon of the products recovered almost exactly equals the total carbon of the reacting substances, showing that all is accounted for, though the products are different from what would have resulted if oxidized separately. Other evidence indicates that the condensation compound is made up of 2 mols of keto acid and 1 of aldehyde, or its derivative. Taking the amount of glycollic acid (12.8 mMols) as an index of the amount of this compound and subtracting the corresponding amounts of glycol aldehyde and of acetoacetate from the totals present, we have the

TABLE VIII.

	Calculated from direct oxidation.	Actually found by analyses.	Difference.
	mMols	*mMols*	*mMols*
CO_2	80.2	52.1	−28.1
Formic acid	37.2	40.1	+ 2.9
Acetic "	40.1	40.1	0
Glycollic " (oxalic after $KMnO_4$)	0	12.8	+12.8

18.6 mMols of glycol aldehyde	=	37.2 milliatoms	C.
40.1 " " acetoacetate	=	160.4 "	"
Total carbon (calculated)	=	197.6 "	"
" " recovered	=	198.0 "	"

TABLE IX.

By direct oxidation:

From glycol aldehyde, 18.6−12.8 = 5.8 × 2 = 11.6 mMols formic acid.

" acetoacetate, 40.1−(12.8 × 2) = 14.5 × 2 = 29.0 " CO_2.

= 14.5 × 1 = 14.5 " acetic acid.

	Total products found.	From direct oxidation.	From condensation compound.	
			12.8 mMols.	Per mol.
CO_2	52.1	29.0	23.1	1.8
Formic acid	40.1	11.6	28.5	2.2
Acetic "	40.1	14.5	25.6	2.0
Glycollic "	12.8	0	12.8	1.0

TABLE X.

Calculated Products per mMol of Condensation Compound from Oxidative Ketolysis with Glycol Aldehyde.

Experiment No.	132*	134*	136	137	138
CO_2	1.9	1.6	1.8	1.9	2.9
Formic acid	2.0	1.1	2.2	1.9	1.3
Acetic "	2.0	2.6	2.0	2.1	2.3
Oxalic "	1.0	1.0	1.0	1.0	1.0

* In Experiments 132 and 134 oxalic acid was not determined, and the calculations are based upon the assumption that the carbon unaccounted for by the volatile acids was present as glycollic acid. This inference is supported by the non-volatile acidity and by the determination of glycollic acid in other experiments.

amounts of each which were presumably oxidized direct without condensation. And by subtracting their known products on direct oxidation from the total products we have the calculated oxidation products of the condensation compound. The balance sheet is then as shown in Table IX.

Making the same calculation for other similar experiments with glycol aldehyde and acetoacetate, the detailed data of which are summarized in Table XI, we find the values shown in Table X.

Although the agreement is not satisfactory, especially in Nos. 134 and 138, in which the yield of acetic acid is high and formic acid low, the results appear to indicate that the oxidation of the condensation compound in the oxidative reaction gives:

2 mols CO_2	From oxidation of 1 mol of condensation product, representing 1 mol of glycol aldehyde + 2 mols of acetoacetate.
2 " formic acid	
2 " acetic "	
1 " glycollic "	

Total of 10 carbon atoms.

With these results from the oxidative reaction we may compare the products from direct condensation of glycol aldehyde and of glyoxal with acetoacetate and subsequent oxidation by alkaline peroxide. The analytical data are given under Experiments 257, 193, and 194 in Table XI. The procedure in all was, in general, as follows: Quantities of approximately neutral solutions of glycol aldehyde or glyoxal and of potassium acetoacetate were mixed in volumetric flasks and stoppered or connected with CO_2 absorption towers. After standing at known temperature for about 24 hours, the solutions were diluted to the mark and aliquots analyzed for CO_2, acetoacetate, and, after dilution and addition of measured amounts of alkali and $BaCl_2$, titrated for total acid formed. Large aliquots were then slowly run into KOH and H_2O_2, and, after the oxidation had continued for the period stated (about 20 hours at 37°C.), the solutions were analyzed for total acid formed (by titration of decrease of alkalinity + base of acetoacetate consumed), CO_2, acetoacetate, total volatile acids (by steam distillation), formic acid, acetic acid (by difference), and oxalic acid (after oxidation of the residue from non-volatile acids by $KMnO_4$). In all experiments blank determinations were made of CO_2, alkali, and volatile acids (acetic) added in reagents and the results corrected by these quantities. The corrected results are given in Table XI.

TABLE XI.

A Summary of Analytical Data, Showing the End-Products of the Ketolytic Reactions of Glycol Aldehyde and Glyoxal in KOH and H_2O_2.

All data are expressed in terms of mMols per liter of solution.

Experiment No.	131	135	132	134	136	138	137	257	193	194
	Glycol aldehyde.		Glycol aldehyde.	Glycol aldehyde.	Glycol aldehyde.	Glycol aldehyde.	Glycol aldehyde.	Glycol aldehyde.	Glyoxal.	Glyoxal.
Aldehyde added	18.6	0	18.6	18.6	18.6	18.6	5.1	23.3	38.5	96.1
Potassium acetoacetate added	0	81.4	40.7	81.4	40.7	40.7	12.2	58.4	102.3	76.7
Direct ketolytic condensation at pH = 7.5 to 8 and = 25°C.										
Acetoacetate consumed								23.0	57.5	
CO_2 liberated								24.9	50.6	59.3
Titratable acid, *cc.* N								31.1	50.5	62.1
Oxidation in 0.5 N KOH + 200 to 400 mMols H_2O_2 at 37°C.										
Incubation, *hrs.*	22	3	22	3.5	18	50	20	17	20	24
Acetoacetate consumed		13.6	40.3	42.1	40.1	34.4	11.8	44.3	70.1	60.1
CO_2	1.0	26.5	53.35	55.65	52.1	55.2	16.5	65.4	109.2	101.3
Formic acid	36.3		37.6	26.3	40.1	28.2	9.9	39.5	55.9	153.4
"Acetic acid"	0		40.9	49.7	40.1	38.4	12.1	43.4	68.2	59.9
Acid equivalent (CO_2 + F. A. + acetic) *cc.* N	38.3		185.2	187.3	184.4	177.0	55.0	213.7	342.5	415.9
Total acids formed (by titration)	38.6	75.0	203.3	204.0	197.0	185.3	57.0	237.4	336.9	422.8
Calculated non-volatile acids	0.7		18.1	16.7	12.6	8.3	2.0	23.7	(−5.6)	6.9
Oxalic acid obtained by $KMnO_4$ oxidation	0		(?)	(?)	12.8	12.2	3.4	15.6	27.5	30.1
Carbon balance	+0.1		−26.1*	−24.3*	+0.2	+3.2	0	−0.9	−0.9	+2.1
Milliatoms C present at start	37.2		198.8	205.6	197.6	181.2	57.4	223.8	357.4	432.6
" " found	37.3		172.7	181.3	198.0	184.4	57.4	222.9	356.5	434.7

It will be noted in this table that from the direct condensation (before oxidation) an amount of CO_2 is formed which almost equals the amount of acetoacetate consumed, and that the total acid formed is roughly equivalent to that due to the CO_2. Thus in Experiment 257 with glycol aldehyde, 23 mMols of acetoacetate disappeared, 24.9 mMols of CO_2 appeared (= 49.8 cc. of N), and the acidity was 54.1 cc. of N (titrated 31.1 + 23 neutralized by base made available by destruction of acetoacetate).

Similarly with the glyoxal experiment, No. 193, 57.5 mMols of acetoacetate disappeared, 50.6 mMols of CO_2 (= 101.2 cc. of N acid) and 108.0 cc. of N total acid were formed. This clearly indicates that each mol of acetoacetate which condenses loses CO_2, and leaves as the residue of the condensation compound a *neutral substance*.

This splitting off of CO_2, however, lags somewhat behind the rate of condensation, as shown by the data in Chart 8. This fact may have considerable importance because of its bearing on the explanation of the reaction.

It will be recalled that in the oxidative reaction also, the CO_2 formed corresponds closely to 1 mol from each mol of acetoacetate; and this production of the same amount in the non-oxidative reaction suggests that it is formed in both cases by decarboxylation, perhaps accelerated in the oxidative reaction by preliminary oxidation of the product.

The total products after oxidation include those from uncombined glycol aldehyde, glyoxal, and acetoacetate, and must be corrected for these amounts, as was done with the results of the oxidative reaction. Since the reactions at which the direct condensation took place were below the alkalinity at which glycollic acid is formed from glyoxal, the amount of oxalic acid found (after $KMnO_4$ oxidation of non-volatile residues) may again be taken as the measure of the condensation compound. And by the same method of calculation we may estimate its products as given in Tables XII, XIII, and XIV. The calculated products of the condensation compound are compared in Table XV. More CO_2 and less formic acid appear to result from the non-oxidative reactions; but the sum of both is about the same in all, 4 of the 10 carbon atoms appearing either as CO_2 or formic acid. The figures for acetic acid are in fair agreement; evidently 1 mol is formed

from each mol of acetoacetate, whether or not it undergoes condensation. Based on these products which account for 8 carbon atoms it seems impossible to decide the path of the oxidative reaction. The other 2 carbons which appear after $KMnO_4$ oxidation as oxalic acid seem to indicate a significant difference. In the

TABLE XII.

Glycol Aldehyde, Direct Condensation with Acetoacetate at pH 7.5 to 8.0 and Subsequent Oxidation by H_2O_2 in 0.5 N KOH. Experiment 257.

	Glycol aldehyde.	Acetoacetate.	Carbon.
	mMols	*mMols*	*milliatoms*
Added	23.3	58.4	
Recovered		14.1	
Oxidized	23.3	44.3	223.8

Oxalic acid (after $KMnO_4$) = 15.6 mMols.

	CO_2	Formic.	Acetic.	Oxalic.	Carbon.
	mMols	*mMols*	*mMols*	*mMols*	*milliatoms*
By direct oxidation of:					
Glycol aldehyde, 23.3 − 15.6 = 7.7		15.4			15.4
Acetoacetate, 44.3 − (15.6 × 2) = 13.1	26.2		13.1		52.4
Total from residual components	26.2	15.4	13.1		67.8
" products found	65.4	39.5	43.4	15.6	222.9
Total from 15.6 mMols condensation compound (by difference)	39.2	24.1	30.3	15.6	155.1
Average per mMol	2.5	1.54	1.94	1.0	9.94

			cc. N
Total carbon present	233.8	Total acid formed (titration)	237.4
" " of products	222.9	" volatile acids	213.7
		Non-volatile acids	23.7
		Oxalic acid (after $KMnO_4$)	15.6

oxidative experiments and also in the direct condensation with glycol aldehyde, the results show an amount of non-volatile acid which approximately corresponds with the amount of oxalic acid found after treatment of the non-volatile residue with alkaline $KMnO_4$. Thus in Experiment 136 (Table XI), the difference between the acid equivalence of the volatile acids and the total

acids formed, as determined by titration of the decrease of alkalinity (+ the base of acetoacetate consumed), is 12.6 cc. of N, while the amount of oxalic acid found is 12.8 mMols. In the direct condensation (Experiment 257), the calculated non-volatile acid is 23.7 cc. of N, and 15.6 mMols of oxalic acid were found. This we interpret to mean that the non-volatile acid which yields oxalic by $KMnO_4$ oxidation is glycollic acid.

TABLE XIII.

Glyoxal, Direct Condensation with Acetoacetate at pH 7.5 to 8 and Subsequent Oxidation by H_2O_2 in 0.5 N KOH. Experiment 193.

	Glyoxal.	Acetoacetate.	Carbon.
	mMols	*mMols*	*milliatoms*
Added	38.5	102.3	
Recovered		32.2	
Oxidized	38.5	70.1	357.4

Oxalic acid (after $KMnO_4$) = 27.5 mMols.

	CO_2	Formic.	Acetic.	Oxalic.	Carbon.
	mMols	*mMols*	*mMols*	*mMols*	*milliatoms*
By direct oxidation from:					
Glyoxal, 38.5 − 27.2 = 11.0		22.0			22.0
Acetoacetate, 70.1 − (27.5 × 2) 15.1	30.2		15.1		60.4
Total from residual components	30.2	22.0	15.1		82.4
" products found	109.2	55.9	68.2	27.5	356.5
From 27.5 mMols condensation compound	79.0	33.9	53.1	27.5	274.1
Average per mMol	2.89	1.23	1.93	1.0	9.97

			cc. N
Total carbon present	357.4	Total acids formed (titration)	336.9
" " of products	356.5	" volatile acids	342.5
		Non-volatile acids	−5.6
		Oxalic acid (after $KMnO_4$)	27.5

From the direct condensation with glyoxal, however, the non-volatile acid is very small in comparison with the oxalic acid found (minus 5.6 cc. of N (Experiment 193) compared with 27.5 mMols of oxalic; and 6.9 cc. of N (Experiment 194) compared with 30.1 mMols of oxalic acid). This appears to indicate that the

TABLE XIV.

Glyoxal, Direct Condensation with Acetoacetate at pH 7.5 to 8 and Subsequent Oxidation by H_2O_2 in 0.5 N KOH. Experiment 194.

	Glyoxal.	Acetoacetate.	Carbon.
	mMols	*mMols*	*milliatoms*
Added	96.1	76.7	
Recovered		16.6	
Oxidized	96.1	60.1	432.6

Oxalic acid (after $KMnO_4$) = 30.1 mMols.

	CO_2	Formic.	Acetic.	Oxalic.	Carbon.
	mMols	*mMols*	*mMols*	*mMols*	*milliatoms*
By direct oxidation of:					
Glyoxal, 96.1 − 30.1 = 66.0		132.0			
Acetoacetate, 60.1 − (30.1 × 2) = 0	0		0		
Total from residual components	0	132.0			132.0
" products formed	101.3	153.4	59.9	30.1	434.7
From 30.1 mMols condensation compound	101.3	21.4	59.9	30.1	302.7
Average per mMol	3.37	0.71	1.99	1.0	10.05

			cc. N
Total carbon present	432.6	Total acids formed (titration)	422.8
" " of products	434.7	" volatile acids	415.9
		Non-volatile acids	6.9
		Oxalic acid (after $KMnO_4$)	30.1

TABLE XV.

Calculated Products from the Oxidation of 1 mMol of Condensation Compound.

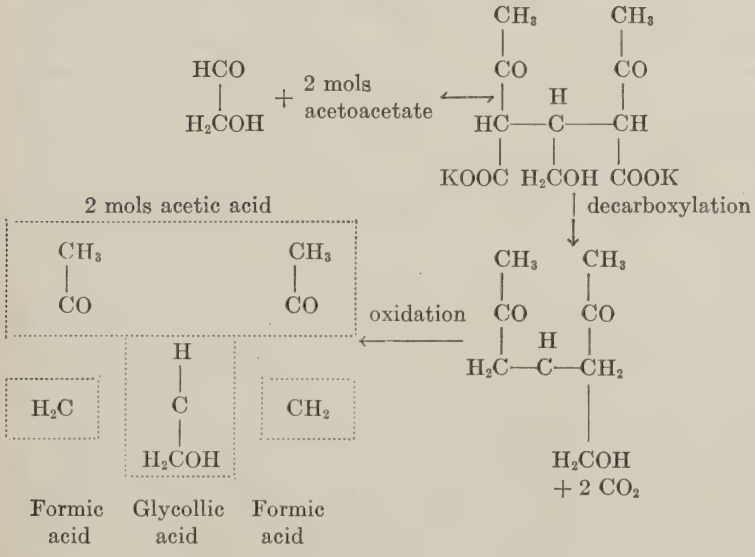
	Glycol aldehyde.		Glyoxal.
	Oxidative ketolysis.	Direct condensation and subsequent oxidation.	Direct condensation and subsequent oxidation.
	Averages.	No. 257	Average of Nos. 193 and 194.
	mMols	*mMols*	*mMols*
CO_2	2.04	2.5	3.13
Formic acid	1.7	1.54	0.97
Acetic "	2.2	1.94	1.96
Oxalic "	1.0	1.0	1.00
Non-volatile acid, *cc.* N	±1.0	1.5	±0.00

non-volatile substance from which the oxalic is formed is *not an acid;* and, if this be so, it constitutes an essential difference in the products of the glyoxal condensation from those from glycol aldehyde. We have not succeeded in identifying the substance. The facts suggest that it may be ethylene glycol.

Since the errors of analysis are accumulated in the calculation of the non-volatile acidity, the data are not highly reliable. The differences are probably beyond the limits of error, however, and we are inclined to accept them as showing a qualitative difference between the reactions.

If this be granted, the conclusion which follows is that *the oxidative ketolytic action of glycol aldehyde does not proceed via glyoxal condensation,*—the same conclusion to which we were led in an earlier section. And if this view be accepted, it seems to prove that the oxidative reaction must result from the condensation with glycol aldehyde itself; *i.e.*, before its oxidation. The products of the oxidation are not inconsistent with this idea. We are inclined, therefore, to suppose that the following outline may represent the course of the oxidative ketolytic reaction of glycol aldehyde.

Scheme III.



The condensation is represented as being reversible, because it is not evident how oxidation could accelerate the reaction otherwise than by removing the product. If the product is not oxidized, and therefore accumulates, it loses CO_2 by decarboxylation and being thus decomposed the condensation continues to take place. In the presence of peroxide, however, the condensation product is more rapidly removed by oxidation, thus allowing rapid condensation to occur. But in the presence of alkali and peroxide the aldose is also being removed by being directly oxidized, and it can react with acetoacetate only to the extent by which the condensation (accelerated by the oxidation of the product) can compete with the direct oxidation. That extensive condensation does take place would seem to prove that it exceeds the aldose oxidation; and the reason that it does so is presumably the greater speed of oxidation of the condensation compound.

According to this explanation, the ketolytic reaction is the resultant of a complex set of dependent or coupled reactions, an exact analysis of which will require a mathematical analysis of the dynamics of the component parts. This we are scarcely in a position to undertake at present.

The extent to which the behavior of glycol aldehyde is applicable to the ketolytic reaction of hexoses we shall take up in a later paper. If, as now seems probable, the ketolytic action of glucose also may be through its direct condensation with acetoacetate, enormously accelerated by oxidation of the product, and, if its antiketogenic action in the body has a similar explanation, one part of our problem is perhaps set aside; namely, the use of the "ketolytic derivative" as a guide in finding the pathway of glucose oxidation. For, in this case, glucose itself would be the ketolytic substance.

If this view proves to be correct for the antiketogenic action of glucose, it would indicate that the same oxidizing mechanism is concerned in the body both with glucose oxidation and the oxidation of its condensation product with acetoacetate, for both fail to occur in severe diabetes and both are restored by insulin. In the test-tube alkali and hydrogen peroxide accomplish the oxidation of both simultaneously. These thoughts suggest the importance of the ketolytic reaction, though other means must be found to judge its direct applicability to physiological phenomena.

SUMMARY.

The investigation of the reaction by which glycol aldehyde, during its oxidation by hydrogen peroxide in alkaline solution, greatly accelerates the rate of oxidation of alkali acetoacetate leads to the conclusion that it is due, not as previously supposed for the ketolytic reaction of hexoses, to a condensation of acetoacetate with an intermediate oxidation product of the sugar, but to a Knövenagel type of condensation of glycol aldehyde itself with acetoacetate, the rate of the condensation being greatly accelerated by the removal of the product by oxidation.

The behavior of glyoxal, the intermediate of glycol aldehyde in its oxidation, is described. Although it condenses with acetoacetate, it probably can be excluded as the "ketolytic" derivative of glycol aldehyde. The rates of condensation and the products of oxidation are given, together with a provisional formula of the condensation product.

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THE EXPERIMENTAL FEEDING OF DRIED BREAST MILK.

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In a previous communication (1) an experimental method for drying breast milk by a modification of the Just-Hatmaker roller process was presented. It had long been felt that could human milk be preserved in some way another step forward towards the solution of the nutritional problems of infancy would be provided (2).

It was found that the drying of the milk was entirely practicable and a powder could be obtained which was completely dehydrated and showed no appreciable change in its chemical composition. The following feeding experiments were undertaken to find out how much of the antiscorbutic value of the milk would be lost by drying and by ageing.

The experimental data which are herewith presented are based on the feeding of young, growing guinea pigs of approximately 250 gm. weight with a standard diet which uniformly produces scurvy in 15 to 20 days, and death in 18 to 25 days. It is the diet recommended by Givens and Cohen (3), Hess (4), and other investigators of these nutritional problems. It consists of a soy bean flour (autoclaved at 20 pounds for 1 hour) to which are added 3 per cent each of dried brewer's yeast, sodium chloride, and calcium lactate. 4 per cent of filter paper is added to give bulk, and the fat-soluble vitamin is supplied by milk, either raw or in the powdered form.

To the basal diet varying quantities of breast milk were added to estimate its antiscorbutic value. The milk was supplied raw or dried. One series included milk which had been freshly dried and another, dried milk which had been kept for 2 years. The results are incorporated in Charts 1 to 4. The experiments were

controlled by a comparable series of guinea pigs fed on the same basal diet but to which cow's milk was added in similar quantities as in the case of the breast milk. Both raw milk and Dryco were used. Finally all the experiments were controlled by a series of animals to which the basal diet alone, and the basal diet plus 10 cc. of orange juice, were given.

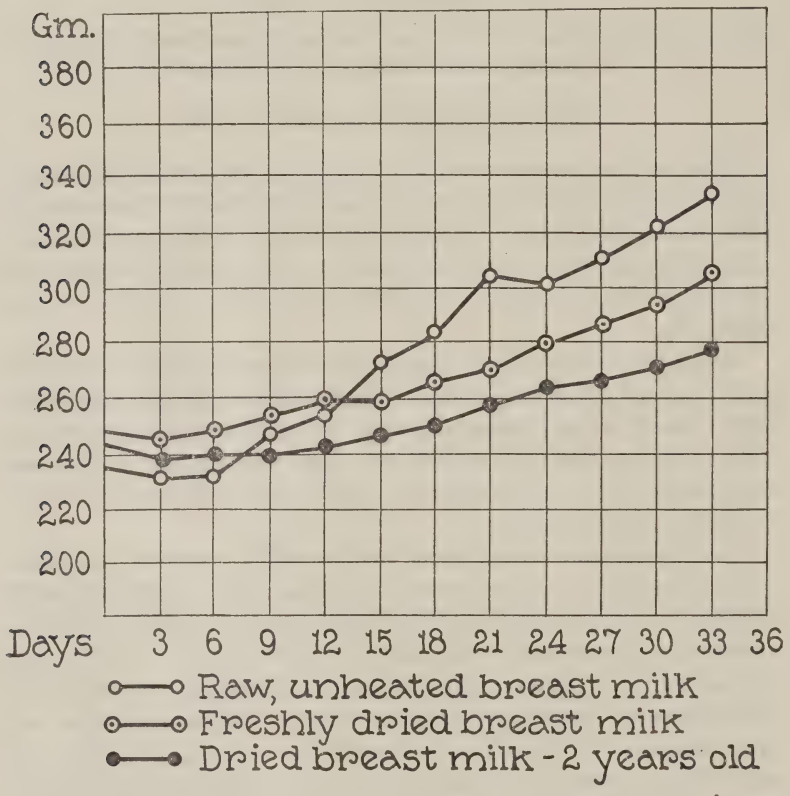

Chart 1. Experiment 1. Basal scorbutic diet plus 60 cc. of breast milk.

All the animals were run in triplicate. The accompanying charts, which show the weight curves of these guinea pigs, represent the average in each instance. All the animals were killed at the end of the experimental period, and a microscopic study of their tissues was made to determine the presence or absence of any scorbutic lesions.

In view of the vast literature on these nutritional experiments, which deals with the methods followed here, it does not seem necessary to enter in detail into the protocols of the individual animals. A brief discussion of the results in each group of cases

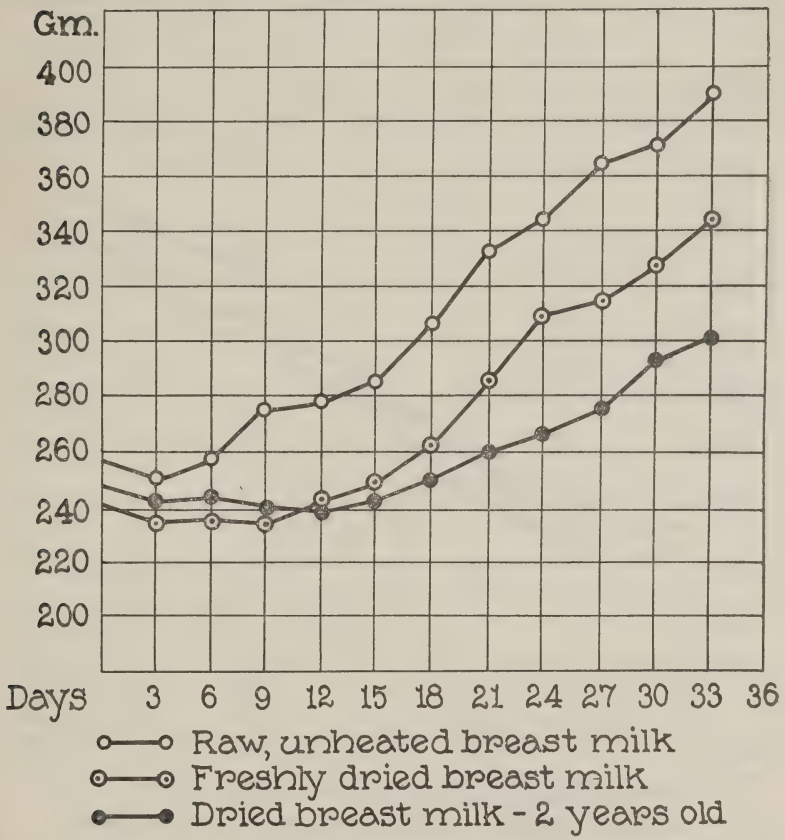

CHART 2. Experiment 2. Basal scorbutic diet plus 120 cc. of breast milk.

is substituted, which, with the corresponding chart, is self-explanatory.

Experiment 1.—Basal scorbutic diet plus 60 cc. of fresh breast milk or its equivalent in dry powder (Chart 1). Breast milk provided (1) raw, (2) freshly dried, and (3) preserved dry for 2 years.

Object of experiment: To note the relative value of minimal amounts of the dried milk products in preventing scurvy.

Results.—With minimal amounts of breast milk the gain in weight is subnormal. This is inversely proportional to the age of the dried milk, although no demonstrable scorbutic lesions are found at the end of a 5 weeks experimental diet period in any of the animals. There has been apparently a further loss of some

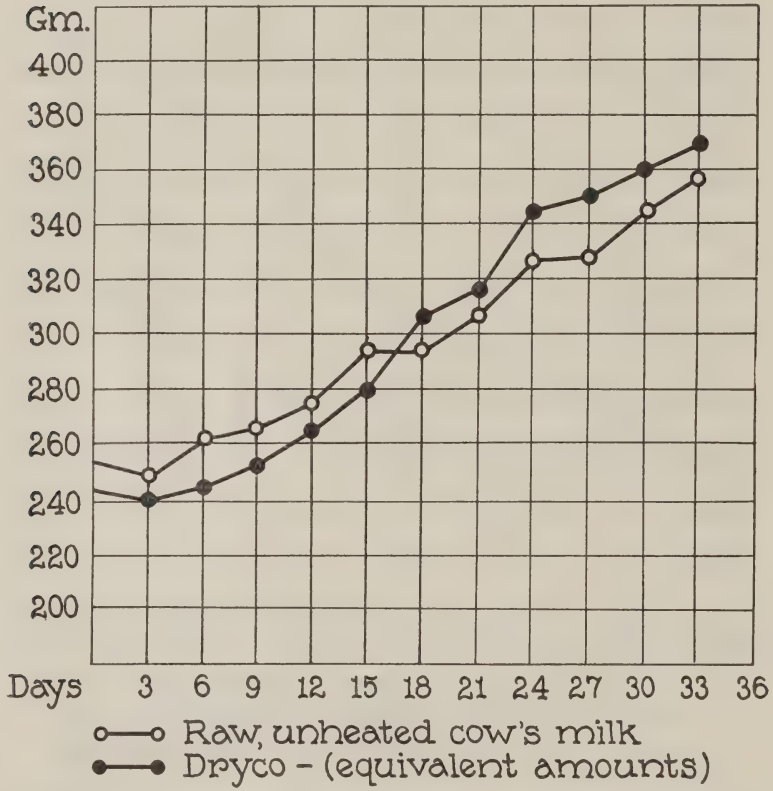

Chart 3. Experiment 3. Control experiment. Basal scorbutic diet plus 100 cc. of cow's milk.

nutritional factor in the drying, which even in the fresh milk is inadequate in the amounts fed, as evidenced by the slow growth curve. This does not have a measurable caloric value, as the diet is in excess of estimated caloric needs of the animals. It must, therefore, be vitaminic in character. We feel that proba-

bly had the animals lived long enough on this diet they would have developed rachitic or scorbutic lesions.

Experiment 2.—Basal scorbutic diet plus 120 cc. of fresh breast milk or its equivalent in dry powder (Chart 2). Breast milk provided, as in previous experiment, raw and powdered.

Object of experiment: To note the relative value of theoretically adequate amounts of the dried milk in preventing scurvy.

Results.—With an adequate amount of fresh milk the gain in weight is normal. With an equivalent amount of the dried milk

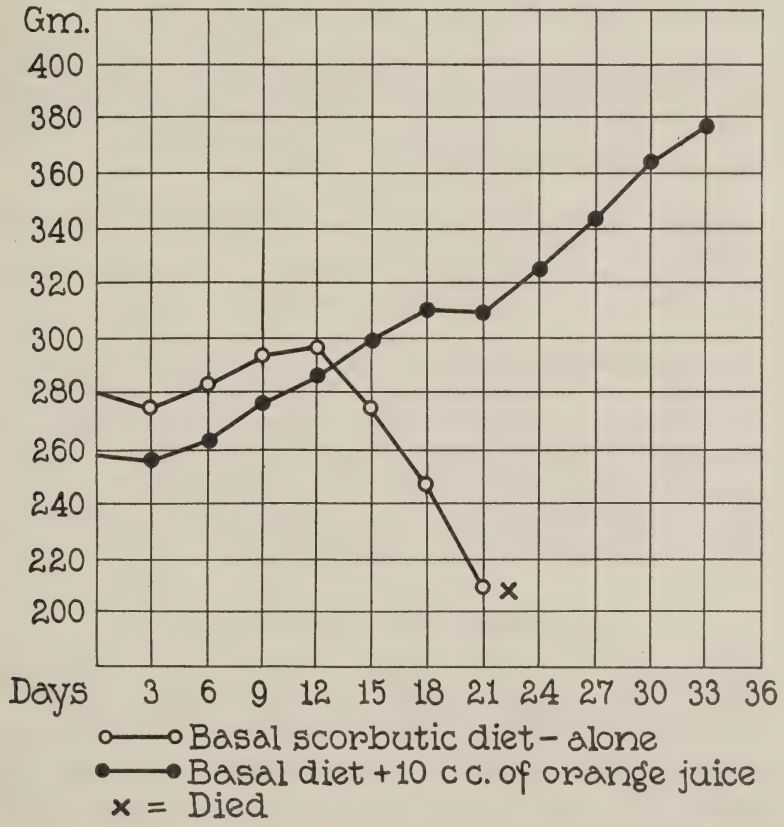

Chart 4. Experiment 4. Control experiment. Basal scorbutic diet alone and with 10 cc. of orange juice.

a somewhat slower gain in weight is noted. This again is felt to be due to partial vitamin deficiency as the caloric values are adequate, and as the gain in weight is slowest in the animals fed on milk powder kept for 2 years. No scorbutic changes are noted in any of the animals during the experimental period of 5 weeks.

Experiment 3.—Basal scorbutic diet plus 100 cc. of cow's milk (Chart 3). Milk supplied (1) raw (certified Walker-Gordon product) and (2) powdered (Dryco from The Dry Milk Co.).

Object of experiment: A control experiment to check the relative value of breast milk as compared to cow's milk in preventing scurvy.

Results.—Normal growth curves are obtained in the control experiments using fresh cow's milk and Dryco in adequate amounts. No scorbutic lesions are noted in the animals at the end of the experimental period of 5 weeks.

Experiment 4.—Basal scorbutic diet alone and with addition of 10 cc. of orange juice (Chart 4).

Object of experiment: A control experiment to prove that the basal diet is deficient only in antiscorbutic vitamin.

Results.—Animals fed the basal diet alone show typical scorbutic lesions within 21 to 25 days. Those on the basal diet in similar amounts with the addition of 10 cc. of orange juice as an antiscorbutic present normal growth curves. These control experiments prove that the diet is adequate and that the slow gain in weight of the animals fed on the dried breast milk is due solely to an inadequate vitamin content.

SUMMARY AND CONCLUSIONS.

The data of feeding experiments utilizing young 250 gm. growing guinea pigs are presented, with their weight curves.

These show that breast milk dried by the method described retains about 80 per cent of its antiscorbutic value when first prepared and about 40 per cent of its original antiscorbutic content after "ageing" for a period of as long as 2 years.

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A QUANTITATIVE STUDY OF SOME ORGANIC CONSTITUENTS OF THE SALIVA.*

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Quantitative studies of the saliva have, in the past, been concerned chiefly with the thiocyanate content and the reaction toward indicators. Comparatively few reports of investigations of the organic constituents are to be found and, of these, very few furnish any quantitative data. This is hardly surprising when one considers that, until the development of the microchemical methods which has proceeded so rapidly in the last decade, the difficulties involved in such a study were well nigh insurmountable.

The first precise quantitative studies of the urea content of the saliva were made by Hench and Aldrich (1) and by Schmitz (2). These investigations were concerned chiefly with the pathological variations of the urea content of the saliva in connection with diagnosis of renal disorders, rather than the physiological variations. This latter phase has been studied by Morris and Jersey (3) in the laboratory of the former who has also recently extended his findings to the study of saliva in disease (4). These studies all agree in demonstrating that the sum of the salivary urea and ammonia nitrogen runs roughly parallel to the blood urea nitrogen, but that it is somewhat less.

One of the earliest studies of the ammonia content of saliva and a most careful investigation which appears to have been overlooked or ignored by subsequent workers, was conducted in 1881 by Heyward (5) in the laboratory of Mallet of the University of Virginia. The figures for salivary am-

*A preliminary report of this work was reported in the Proceedings of the Society for Experimental Biology and Medicine (Lewis, H. B., and Updegraff, H., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 168).

†An abstract of a thesis submitted by Helen Updegraff in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the University of Michigan.

monia reported, 4.0 to 10.0 mg. per 100 cc. of saliva, agree very closely with the figures found by recent investigators (1, 2, 3). Hench and Aldrich (1) and Schmitz (2) reported figures for salivary urea and ammonia in normal and pathological individuals. They have found that the sum of the urea and ammonia nitrogen of the saliva closely approximates the urea nitrogen of the blood. Moreover, they have observed that, in urea retention, the sum of the urea and ammonia nitrogen of the saliva always increases with an increase in the blood urea nitrogen. They conclude, therefore, that the sum of the urea and ammonia nitrogen of saliva is a valuable index of renal functional capacity. It should be mentioned in this connection that Schmitz satisfied himself, by a series of careful experiments, that the salivary ammonia owes its origin to bacterial action upon the urea normally present in the saliva.

Earlier analyses of the uric acid content of the saliva (6, 7, 8) are open to criticism because of the methods employed. In 1919, Lowenstein and Gies (9) found an average uric acid content of 2.10 mg. per 100 cc. for men, and for women, 1.11 mg. per 100 cc. They also succeeded in separating uric acid in crystalline form from saliva. Morris and Jersey (3) obtained values for uric acid similar to those of Lowenstein and Gies in salivas collected after the chewing of paraffin. They believed, however, that the saliva obtained as the natural secretion of resting glands was much more uniform in its content of uric acid and that a correct picture of salivary secretion was not obtained after paraffin chewing. No reliable figures for the non-protein nitrogen of the saliva are available. The amino acid and creatinine content of saliva have been studied by Morris and Jersey (3).

The progress in the knowledge of the organic constituents of the saliva that has been made indicates the need for more comparative data on the organic constituents of blood and saliva. In obtaining such comparative data it is desirable to use, as far as possible, the same methods for saliva as for blood. We have studied the comparative composition of the saliva and blood of normal individuals with reference to the non-protein nitrogen, urea, ammonia,[1] and uric acid. In all cases an attempt has been made to utilize the standard Folin-Wu procedure for blood or to so modify it as to make it of value for the analysis of saliva. Observations which confirm and extend the work of other investigators as to the origin and significance of the ammonia of the saliva have also been made. Certain differences in the amount of the "undetermined non-protein nitrogen" in the blood and in saliva are considered.

[1] It should be mentioned that no determinations of blood ammonia were made in this investigation. Even under pathological conditions the ammonia of the blood (10) amounts to only a few hundredths of a milligram per 100 cc., an amount which is negligible for all practical purposes.

EXPERIMENTAL.

General Procedure.

The subjects of the experiments were healthy men and women, students or laboratory workers for the most part. The sample was collected from 1 to $1\frac{1}{2}$ hours after a light breakfast. Each subject was first required to rinse the mouth thoroughly with water. Paraffin was chewed and the first few cubic centimeters of saliva were rejected. From 30 to 35 cc. of saliva were then collected, the time required for collection of the sample was noted, and the analysis carried out immediately. When comparative studies were made on the blood, 5 to 10 cc. of blood were drawn from an arm vein. The blood was always drawn immediately upon the conclusion of the period during which the saliva was collected.

Precipitation of Protein.

Before a workable scheme of analysis could be devised for the various organic constituents of saliva, it was desirable to find a simple efficient method for the removal of the protein, comparable to the admirable method of Folin and Wu for blood. A few preliminary trials showed that the Folin-Wu method, as it is applied to blood, is not adapted to the removal of protein from saliva. The filtrations were slow and tedious and the filtrates thus obtained were turbid and unsuited for the various determinations. In the search for a suitable method, all the ordinary protein precipitants were studied. The only combination that effected satisfactory and complete removal of the protein was that of trichloroacetic acid and kaolin. This was, however, open to the objection that the trichloroacetic acid filtrate can be used for the determination of comparatively few constituents.

It seemed, therefore, worth while for a time to persist in the effort to apply the precipitation method of Folin and Wu to saliva. It was found that the salivary protein could be completely precipitated by tungstic acid in the presence of a very small amount of calcium chloride, followed by treatment with kaolin. A further difficulty presented itself when it was attempted to use this filtrate for the determination of uric acid by the recent method of Benedict (11). When the filtrate was made alkaline with sodium cyanide, the solution became quite turbid, due probably

to the precipitation of calcium salts, and the cloudiness was not dissipated upon addition of the arsenophosphotungstic acid reagent. It was then decided to remove the excess calcium by precipitation with a slight excess of oxalic acid. However, since, after addition of the oxalic acid, the pH of the solution was much too low for complete precipitation of calcium as the oxalate (12), it was necessary to correct the pH, by addition of alkali, to the range 4.0 to 5.6. By the use of brom-phenol blue, an indicator efficient within this range of pH, the amount of alkali necessary to raise the pH to the desired value was determined. After the addition of the proper amount of alkali, the filtrate obtained was found to be entirely suitable for the determination of uric acid by the method of Benedict, as well as for the other determinations made.

The method of precipitation which has been found to yield a filtrate adapted to the largest number of determinations is as follows:

To 25 cc. of saliva in a 150 cc. Erlenmeyer flask are added 11.6 cc. of water, 2.5 cc. of 10 per cent sodium tungstate, and slowly, while shaking, 2.5 cc. of 0.66 N sulfuric acid. 2 cc. of 10 per cent calcium chloride are then added and the mixture is shaken; 5 cc. of 5 per cent oxalic acid and lastly 1.4 cc. of 10 per cent sodium hydroxide are then added, making a total volume of 50 cc. A rubber stopper is inserted in the mouth of the flask and the mixture is well shaken and allowed to stand for from 5 to 10 minutes. About 2 gm. of kaolin are added and the flask is shaken vigorously a few times. The mixture is poured upon a pleated hardened filter (Schleicher and Schüll, No. 575 is of satisfactory quality) and the funnel is covered with a watch-glass during the filtration which usually proceeds rapidly. The filtrates are often water-clear from the first drop, but should the first 5 or 10 cc. of filtrate be turbid, they should be returned to the filter. The method of precipitation is quite elastic in that much less saliva may be used and the amount of water correspondingly increased. In fact, this is often an advantage as when less saliva is used the speed of filtration is increased. Occasionally one encounters a saliva that filters very slowly and it will then be necessary to use a higher dilution. However, higher dilutions are to be avoided, as a rule, as some of the constituents (*e.g.* uric acid and ammonia) are often present in such minute quantities that accurate determinations cannot be made when the saliva is diluted much more than 1:1

The filtrates obtained by this method are faintly acid to litmus, do not give the biuret test, and are, apparently, entirely free from protein. On standing for 24 hours at room temperature the value

for ammonia in such filtrates remains unchanged, indicating that, once the protein is removed from saliva by this method, no further hydrolysis of the urea occurs. Moreover, the value for uric acid in these filtrates does not change appreciably on standing 24 hours at room temperature. In three experiments the uric acid values immediately after precipitation of the protein were 1.3, 1.1, and 1.1 mg. per 100 cc. After the filtrates had stood 24 hours at room temperature the values were 1.0, 1.03, and 1.0 mg., respectively, variations within the experimental error of the method.

Methods of Analysis.

The protein-free filtrates were analyzed as follows: non-protein nitrogen and glucose, by the method of Folin and Wu: urea and ammonia by the aeration urease method of Folin; and uric acid by the recent modification of Benedict (11). The recovery of pure substances added to the saliva was satisfactory in every case.

In view of the fact that most of the ammonia and urea determinations on saliva reported by others (1, 2) have been conducted upon the secretion without subjecting it to any preliminary treatment, it was deemed advisable to compare the values obtained for urea and ammonia nitrogen in the filtered saliva with those obtained in aliquots of the modified Folin-Wu filtrate from the same sample. The values obtained from the filtered saliva agreed with those obtained from the Folin-Wu filtrate practically within the experimental error of the method as borne out by the following typical data: ammonia nitrogen, 2.6 and 2.7 mg., ammonia plus urea nitrogen, 7.1 and 6.8 mg., urea nitrogen 4.5 and 4.1 mg., per 100 cc. in the Folin-Wu filtrate and untreated saliva, respectively.

DISCUSSION.

When it was satisfactorily demonstrated that the various organic constituents, mentioned above, could be accurately determined in the saliva by the technique just outlined, a large number of normal salivas were analyzed in order to gain some idea of the range of normal values. In most of the salivas examined the total solids were determined to find whether there was any evident relation between solids and the amount of any of the organic constituents.

No such relation could be established, however. In Table I is presented a summary of all the salivary analyses made in this investigation with the range through which the amounts of each constituent varied.

The results of comparative studies of the blood and saliva are presented in Tables II and III. The data in Table III include

TABLE I.

Summary of Analyses of Saliva.

The figures represent milligrams per 100 cc. of saliva.

Constituent.	No. of subjects.	No. of specimens.	Range.	Average.
Men.				
			mg.	*mg.*
Total solids	52	66	386.0–840.0	597.0
Non-protein nitrogen	50	62	7.0– 26.7	13.5
Ammonia nitrogen	27	31	2.1– 13.2	6.1
" plus urea nitrogen	14	18	8.5– 15.9	11.5
Urea nitrogen	14	16	0.0– 6.7	4.2
Uric acid	55	72	0.5– 2.9	1.7
Women.				
Total solids	17	27	406.0–860.0	544.0
Non-protein nitrogen	17	19	5.6– 24.4	11.6
Ammonia nitrogen	7	7	2.8– 8.3	4.6
Uric acid	17	27	0.6– 2.4	1.5
Totals—men and women.				
Total solids	69	93	386.0–860.0	581.0
Non-protein nitrogen	67	81	5.6– 26.7	13.0
Ammonia nitrogen	34	38	2.1– 13.2	5.7
" plus urea nitrogen	15	19	6.8– 15.9	11.3
Urea nitrogen	15	17	0.0– 6.7	4.1
Uric acid	72	99	0.5– 2.9	1.6

also urea, the ammonia plus urea nitrogen, and the residual non-protein nitrogen of the saliva and blood; *i.e.*, the nitrogen not present as urea, ammonia, or uric acid. An inspection of these tables shows that the values for the various organic constituents of saliva are considerably lower than the corresponding values for the blood. This difference is most marked in the case of the non-

TABLE II.

Determination of Total Solids, Ammonia Nitrogen, Non-Protein Nitrogen, and Uric Acid in the Saliva of Fifteen Normal Men and Seven Normal Women and Simultaneous Determinations of Non-Protein Nitrogen, Uric Acid, and Sugar in the Blood of Thirteen Men and Five Women.

The figures represent milligrams per 100 cc. of saliva and blood.

Subject.	Total solids.	Ammonia nitrogen.	Non-protein nitrogen.		Uric acid.		Sugar.	
	Saliva.	Saliva.	Saliva.	Blood.	Saliva.	Blood.	Saliva.	Blood.
				Men.				
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
W. G.	734	7.1	18.6	37.2	1.7	3.2	None.	80
G. H.	570	4.0	11.1	37.3	1.8	4.1	"	93
M. T.	668	5.9	14.3	42.1	1.3	4.4	"	87
R. J.	602	3.1	13.9	35.9	2.0	4.3	"	81
D. M.	496	4.2	14.7	34.1	1.4	4.1	"	83
E. W.	644	2.8	10.2	33.4	2.2	4.3	"	78
C. W.	724	4.4	17.0	36.6	1.7	3.0	"	76
H. D.	564	2.3	10.5	35.9	1.7	3.5	Slight reduction.	84
L. H.	722	5.6	10.0	31.4	1.3	3.9	None.	89
D. D.	704	8.1	15.0		1.7		Slight reduction.	
R. C.	536	3.5	15.0		1.8		None.	
J. B.	506	7.3	13.1	39.4	0.8	3.3	"	92
H. L.	522	7.0	12.0	33.3	2.1	4.1	"	95
W. B.	386	4.0	11.5	35.1	0.7	3.4	Very slight reduction.	80
P. S.	442	2.1	10.9	35.2	0.9	2.6	None.	70
				Women.				
W. W.	476	5.0	10.7	32.0	1.1	2.9	None.	86
E. S.	578	3.8	9.8	31.4	1.2	2.8	"	81
S. L.	506	4.6	9.3	32.7	1.2	2.8	"	91
E. T.	412	3.8	8.3		0.6		"	
M. W.	412	2.8	9.6	33.3	2.0	4.6	"	106
M. M.	508	8.3	15.5		1.0		"	
H. U.	490		10.3	28.0	1.5	4.1	"	106

protein nitrogen and uric acid and least in the case of the urea-ammonia nitrogen fraction. In order to indicate more definitely the quantitative relationship between the individual constituents of these two fluids, the values for the saliva in Table III have been

TABLE III.

Determinations of Non-Protein Nitrogen, Ammonia Nitrogen, Urea Nitrogen, Uric Acid, and Residual Non-Protein Nitrogen in the Saliva and Simultaneous Determinations of Non-Protein Nitrogen, Urea Nitrogen, Uric Acid, and Residual Non-Protein Nitrogen in the Blood of Fifteen Normal Individuals.

The figures represent milligrams per 100 cc. of saliva and blood.

Subject.	Non-protein nitrogen.		Ammonia nitrogen.	Ammonia plus urea nitrogen.	Urea nitrogen.		Uric acid.		Residual non-protein nitrogen.			
	Saliva.	Blood.	Saliva.	Saliva.	Saliva.	Blood.	Saliva.	Blood.	Saliva.		Blood.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent**	*mg.*	*per cent**
J. J.	15.8	37.0		13.5		15.0	0.5	3.2	2.1	13.5	20.9	56.6
	10.8	40.5		10.4		14.4	1.5	3.6	−0.1	−0.9	24.9	61.5
	14.8	42.1	13.2	13.2	0.0	16.7	0.9	3.8	1.3	8.8	24.1	57.3
	11.7	37.4	5.9	10.4	4.5	15.9	1.3	3.9	0.9	7.4	20.2	54.0
P. B.	14.6	37.0	6.9	12.2	5.3	14.6	1.7	3.2	1.8	12.5	21.3	57.7
G. P.	14.7	39.9	9.9	13.6	3.7	14.7	1.7	3.5	0.5	3.6	24.0	60.2
R. C.	11.0	34.8	4.0	10.0	6.0	15.6	2.5	4.4	0.2	1.6	17.7	51.0
C. P.	11.4	35.4	6.4	10.2	3.8	14.9	1.6	3.2	0.7	6.1	19.4	54.9
P. J.	15.8	35.6	5.7	11.1	5.4	16.7	1.8	3.5	4.1	26.0	17.7	49.8
T. T.	20.3	37.5	7.5	8.5	1.0	12.5	1.6	4.8	11.3	55.5†	23.4	62.4
J. B. U.	13.4	32.2	6.6	8.8	2.2	10.2	1.1	3.2	4.2	31.6	20.9	65.0
L. F.	19.6	42.3	10.8	15.9	5.1	21.8	1.4	4.1	3.2	16.5‡	19.1	45.2
A. C.	13.2	35.3	5.8	9.9	4.1	15.2	1.2	2.6	2.9	22.0	19.2	54.5
G. L.	13.8	37.3	6.0	11.9	5.9	18.9	0.9	3.3	1.6	11.6	17.3	46.4
M. L.§	8.2	25.3	4.2	6.8	2.6	9.6	1.0	2.8	1.1	13.2	14.8	58.4
H. E.	19.1	33.9	9.0	13.5	4.5	13.0	1.2	4.1	5.2	27.2	19.5	57.6
	18.8	40.8	5.3	12.0	6.7	14.7	1.8	4.0	6.2	33.0	24.8	60.7
H. L.	12.2	36.7	7.3	11.5	4.2	14.5	1.8	4.0	0.1	0.8	20.9	56.9
D. Mc.	12.0	29.9	5.7	10.9	5.2	14.8	2.7	2.9	0.2	1.7	14.1	47.3

*That is, per cent of non-protein nitrogen.

†Saliva contaminated with a little blood. Note high non-protein nitrogen.

‡Saliva contained some food particles. Note high non-protein nitrogen.

§Woman.

recalculated in terms of the percentage of the corresponding constituent of the blood of the same individual and are recorded in Table IV. In addition, all the comparative figures obtained for blood and saliva have been summarized in Table V.

68 of the salivas analyzed were tested for sugar by the Folin-Wu method. In Table II where figures for blood sugar are recorded, the reaction of the corresponding salivary filtrate to the Folin-Wu reagent is also recorded. A decided reduction was noted in a few of the 68 salivas examined, but the color developed was never of sufficient intensity to permit a quantitative estima-

TABLE IV.

Figures for Saliva from Table III Expressed as Percentage of the Corresponding Constituent in the Blood of the Same Individual.

Subject.	Non-protein nitrogen.	Ammonia plus urea nitrogen as percentage of blood urea nitrogen.	Urea nitrogen.	Uric acid.	Residual non-protein nitrogen.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
J. J.	42.7	90.0		15.6	10.1
	26.7	72.2		41.7	0.0
	35.2	79.0	0.0	23.7	5.4
	31.3	65.4	28.3	33.3	4.5
P. B.	39.5	83.6	36.3	53.1	8.5
G. P.	36.8	92.5	25.2	48.6	2.1
R. C.	31.6	64.1	38.5	56.8	1.1
C. P.	32.2	68.5	25.5	50.0	3.6
P. J.	44.4	66.5	32.3	51.4	23.2
T. T.	54.1	68.0	8.0	33.3	48.3
J. B. U.	41.6	86.3	21.6	34.4	20.1
L. F.	46.3	72.9	23.4	34.2	16.8
A. C.	37.4	65.1	27.0	46.2	15.1
G. L.	37.0	63.0	31.2	27.3	9.3
M. L.*	32.4	70.8	27.1	35.7	7.4
H. E.	56.3	103.9	34.6	29.3	26.7
	46.1	81.6	45.6	45.0	25.0
H. L.	33.2	79.3	29.0	45.0	0.5
D. Mc.	40.1	73.7	35.1	93.1	1.4

*Woman.

tion of the amount of reducing substance present. Nevertheless, in these cases the color developed was deeper than that developed in a blank control containing only the reagents. In the case of three normal individuals the administration of from 100 to 300 gm. of glucose produced a decided hyperglycemia, but no increased amount of glucose was eliminated in the saliva 1 hour after the glucose administration. In view of these results and those of

numerous other investigators, it is probably safe to conclude that glucose, in significant amounts, is not a normal constituent of saliva.

The average normal figures for salivary non-protein nitrogen give, it is believed, an accurate idea of the actual average normal value for this constituent. However, the maximum values (26.7 mg. per 100 cc. for men and 24.4 mg. per 100 cc. for women) obtained for non-protein nitrogen are probably really higher than the actual normal. Very few values were found in the neighborhood of these maximum values and, invariably, when high figures

TABLE V.

Summary of the Comparative Data on Blood and Saliva.

Constituent.	No. of subject.	No. of specimens	Blood.		Saliva.		Saliva.*	
			Range.	Average.	Range.	Average.	Range.	Average.
			mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
Non-protein nitrogen.	31	37	25.3–42.3	35.5	8.2–20.3	13.2	26.7– 56.3	37.0
Ammonia plus urea nitrogen	15	19	9.6–21.8	14.9	6.8–15.9	11.3	63.0–103.9	76.1
Uric acid	31	41	2.6– 4.8	3.6	0.5– 2.7	1.5	15.6– 93.1	40.1
Residual non-protein nitrogen	15	19	14.1–24.9	20.2	0.0–11.3†	2.5	00.0– 48.3	12.1

*In these two columns the figures for saliva are expressed as percentages of the corresponding constituent in the blood of the same individual.

†Compare foot-note 2 to text.

were obtained, there had been opportunity for some decomposition in the sample; *e.g.*, standing in a warm room for an hour or so before it was possible to precipitate the protein. The very low figure (5.6 mg.) was obtained in only one case and may possibly be ascribed to an experimental error.

It will be observed that the values for ammonia nitrogen show a wide range of variation probably depending upon the extent of bacterial decomposition of salivary urea. The fact that the values for urea nitrogen also showed a similar range of variation is in accord with this idea. The urea nitrogen may even be reduced to zero. Undoubtedly this value also depends upon the extent of

bacterial decomposition that has taken place. This matter will be discussed later in more detail. As already pointed out by Hench and Aldrich (1) and Schmitz (2), the value for ammonia plus urea nitrogen maintains a much greater constancy than the urea nitrogen and is, therefore, to be regarded as more significant than the value for either urea or ammonia nitrogen alone.

The value for uric acid in the saliva varied from 0.5 to 2.9 mg. with an average of 1.6 mg. per 100 cc. It is believed that this figure is an accurate estimate of the average normal value. Very few samples were found to run as high as 2.5 mg. per 100 cc. and the low figure, 0.5 mg., was obtained in only one instance. A study of the salivary uric acid of one normal woman over a period of several weeks indicated that a relatively constant level was maintained in the secretion. The extreme values were 0.9 and 1.7 mg. per 100 cc. with an average value of 1.2 mg. and a maximum deviation from the mean of 0.5 mg.

In agreement with Schmitz we found that the figure for the sum of the urea and ammonia nitrogen approached quite closely the value for the urea nitrogen of the corresponding blood (Table IV). However, in the fifteen individuals in which simultaneous analyses were made of the blood and saliva, the sum of the urea and ammonia nitrogen of the saliva averaged 76.1 per cent of the urea nitrogen of the blood, a value somewhat lower than the average values, 89.4 per cent, reported by Schmitz (2) and 80 per cent by Hench and Aldrich (1). In this connection it is of interest that Myers and Fine (13) have found that the urea of spinal fluid amounts to 88 per cent of that of the blood. In this respect, saliva may be said to resemble spinal fluid.

A consideration of the total average values for the various organic constituents of saliva (Table I) brings out another interesting point which merits discussion here. The average figure for salivary non-protein nitrogen is 13.0 mg. per 100 cc. and for salivary urea plus ammonia nitrogen, 11.3 mg. per 100 cc. Thus in saliva the urea plus ammonia nitrogen comprises about 86.9 per cent of the non-protein nitrogen. This is in marked contrast to blood in which, as a rule, the urea nitrogen amounts to less than 50 per cent of the non-protein nitrogen. Moreover, the average value for salivary uric acid is 1.6 mg. per 100 cc. or, expressed in terms of uric acid nitrogen, 0.53 mg. per 100 cc. Thus the nitro-

gen of the urea, ammonia, and uric acid amounts to 11.83 mg., or 91 per cent of the non-protein nitrogen. There remains only 9 per cent or about 1 mg. unaccounted for.[2] It is difficult to reconcile these results with the figures for salivary amino acid nitrogen reported by Morris and Jersey (3). These investigators found from 4 to 11 mg. and, as a rule, about 7 or 8 mg. of amino acid nitrogen per 100 cc. of saliva, while our results would indicate that there can be no significant amounts of amino acid nitrogen in this secretion. Saliva thus appears to resemble urine more than blood in that the urea plus ammonia nitrogen accounts for the greater part of the non-protein nitrogen.

The failure to obtain evidence of any significant amount of nitrogen not accounted for as urea, ammonia, and uric acid nitrogen in most of the salivas examined led us to subject our experimental procedure to careful critical study in order to determine whether losses in amino acid nitrogen by precipitation or otherwise has occurred. Morris and Jersey (3) have reported considerable amounts of amino acid nitrogen in saliva and noted that the quantity present in saliva obtained after paraffin chewing was greater than that in saliva from the resting glands. We were unable to remove the ammonia from our filtrates by permutit and were accordingly unable to utilize the Folin method for amino acids. Morris and Jersey state that the application of the Folin-Wu methods to saliva proved entirely satisfactory, but do not give details as to how the amino acid method was applied. The method of removal of protein used by them has not, in our hands,

[2] Attention should be called to the fact that the highest residual non-protein nitrogen figure observed (Subject T. T., Table III) was obtained from a saliva which contained a sufficient amount of blood to permit of the recognition of its presence by the faint color. Since blood contains a much greater amount of residual non-protein nitrogen than saliva, this unusually high value for residual non-protein nitrogen in the saliva is presumably due, to some extent, at least, to the presence of blood. In most other cases in which high figures for saliva were obtained, the salivas were collected on warm days and stood for an hour or more before precipitation of the protein so that autolysis between the time of collection and of deproteinization probably accounts for the high residual non-protein nitrogen. Our decomposition experiments with saliva have shown that this fraction of nitrogen increases if salivas are allowed to stand and its formation is not checked as is the ammonia formation by the presence of a preservative (chloroform).

yielded a filtrate from which the considerable amounts of ammonia present could be removed by permutit prior to the amino acid determination. It seemed possible that the method of precipitation developed by us might precipitate also the greater part of the amino acids. A solution of alanine, glycine, and aspartic acid was prepared containing 300 mg. of nitrogen per liter, 100 mg. of nitrogen being derived from each of the three acids. Known amounts of this amino acid mixture were added to saliva and the non-protein nitrogen was determined in the filtrates from the original saliva and from the saliva to which the amino acid mixture had been added. Casein was digested by trypsin, and further hydrolyzed by boiling with hydrochloric acid until the biuret reaction was negative. This mixture of amino acids was added to saliva

TABLE VI.

Source of amino acids added.	Nitrogen added as amino acids.	Salivary non-protein nitrogen found.	Extra nitrogen recovered.
	mg.	*mg.*	*mg.*
	0.0	12.54	
Pure glycine, alanine, and aspartic acid.	12.0	24.86	12.32
	0.0	9.41	
Tryptic digest of casein completely hydrolyzed.....	10.0	18.31	8.90

and the non-protein nitrogen content of the filtrates determined as in the previous experiments. Typical data are recorded in Table VI. In all cases, the added nitrogen was satisfactorily recovered, thus demonstrating that under the conditions with which we were concerned, significant amounts of nitrogen were not precipitated. Losses of amino acids in our method of deproteinization can hardly explain the discrepancy between our figures for "residual" non-protein nitrogen and those of Morris and Jersey for amino acid nitrogen.

We also carried out a series of experiments in which the Folin amino acid procedure was used with the salivary filtrates without any attempt to remove the ammonia. Both glycine and ammonium sulfate were used as standards for comparison with the unknowns. In every case, the color of the unknown was pink,

similar to the color of the ammonium sulfate standard rather than to the yellow of the glycine standard. Moreover, when the ammonium sulfate standard was used as a basis of calculation, the results showed that there was present in the unknown practically no nitrogen which reacted with the reagent except that which could be accounted for by the ammonia content as determined by aeration. We believe that these experiments demonstrate that amino acid nitrogen is present in saliva in very small amounts, if at all.

The question of the nature and extent of the decomposition occurring in saliva on standing at room temperature seemed to be of sufficient importance to justify a few experiments. Salivas to which, in some instances, urea had been added were incubated at room temperature for a period of from 4 to 24 hours, both with and without chloroform. The changes in the modified Folin-Wu filtrate from saliva on standing were also studied. The more important results of the experiments may be briefly summarized. There is a progressive increase in the non-protein nitrogen, ammonia plus urea nitrogen, and ammonia nitrogen of the saliva when allowed to stand at room temperature without a preservative. A definite decrease in the urea nitrogen is associated with the increase in ammonia nitrogen. Urea added to saliva is also converted to ammonia on standing without a preservative. The percentage of urea hydrolyzed in 24 hours varied in these experiments from 35 to 88 per cent. The cause of this variation is not known, but it would seem that differences in the reaction of the saliva, its urea content, and possibly the bacterial flora may be factors concerned. The values for ammonia and non-protein nitrogen in the modified Folin-Wu filtrate do not change in 24 hours at room temperature.

The presence of chloroform effectually checks the increase of ammonia, but has no influence on the increase in the non-protein nitrogen. The increase in this latter constituent is probably not due to bacterial action, but to the action of autolytic enzymes of the cells, since it is not checked by the presence of chloroform.

These and other similar experiments supplement and extend the earlier work of Severin (14) who concluded that ammonia was probably formed in the mouth by bacterial action upon other nitrogenous substances and of Hench and Aldrich (1) and of Schmitz (2) who showed that the ammonia was formed largely

at the expense of urea. They emphasize the need of prompt analysis if a true picture of the composition of the saliva as secreted is desired.

In conclusion attention should be called to the fact that the figures obtained in this study represent analyses of saliva collected after one type of stimulation only, paraffin chewing. For the purposes of the present study, comparative analyses of blood and saliva, it was felt that a uniform procedure should be adopted. Whether a more typical picture of salivary secretion would be shown by salivas obtained by other types of stimulation or by the secretion of the resting gland must be determined by further work.

SUMMARY.

1. A modification of the Folin-Wu blood method for the precipitation of protein from saliva is described. In the filtrates obtained, non-protein nitrogen, urea, ammonia, uric acid, and glucose have been determined by the usual methods.

2. A comparison of the composition of the blood and saliva of the same individual indicates that the non-protein nitrogen of saliva averages 37.0 per cent of that of the blood; the ammonia plus urea nitrogen, 76.1 per cent; the uric acid, 40.1 per cent; and the residual non-protein nitrogen, 12.1 per cent. Of these constituents of the saliva the ammonia plus urea nitrogen most nearly equals the urea nitrogen of the blood.

3. The relatively small quantity of residual non-protein nitrogen of the saliva in most cases would indicate that amino acid nitrogen must be present in saliva in very small amounts, if at all. The activity of the salivary glands would appear to be selective, since of the blood constituents, glucose and amino acids are not secreted in significant amounts in the saliva, while urea and uric acid readily pass into the secretion.

4. The ammonia in saliva originates largely from the decomposition of urea. This formation of ammonia may be checked by the presence of chloroform.

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SOME CHEMICAL CHANGES IN THE BLOOD OF DOGS AFTER THYROPARATHYROIDECTOMY.

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In connection with other investigations in this laboratory, a number of dogs were subjected to thyroparathyroidectomy. It was thought advisable to secure samples of blood from several of these at different times and to analyze this blood for various constituents. This paper will report the results of such analyses.

Methods.

The thyroparathyroidectomies were performed under ether anesthesia. In many of the experiments, blood was drawn from the external jugular vein at that time. The results obtained upon the analysis of that blood are summarized in the tables under the heading "At operation." About 28 or 30 hours later, after the thoroughness of the parathyroidectomy had been established by observation of the decrease in the ratio of phosphorus to nitrogen in the urine (1, 2, 3), other samples of blood were drawn from the femoral artery, using cocaine anesthesia. The figures obtained appear in the columns headed "Pretetanic." After tetany appeared, blood was drawn from the other femoral artery. Three dogs did not show the retention of phosphorus and did not develop tetany and in three others blood was obtained, under cocaine anesthesia, without previous operative interference.

The blood was received in 15 cc. centrifuge tubes, containing 50 mg. of potassium oxalate, dried on the sides, and was immediately centrifuged to obtain the plasma. Another, smaller, sample of blood was allowed to clot.

The analytical methods used were the following:

Titratable alkali.....Greenwald and Lewman (4)
Calcium.....Kramer and Tisdall (5)
Sugar.....Benedict (6), Folin and Wu (7)
Nucleotide nitrogen.....Jackson (8)
Inorganic phosphate.....Briggs' modification of the method of Bell and Doisy (9)
Total "acid-soluble" phosphorus.....As much of the trichloroacetic acid filtrate as remained (generally the equivalent of from 1 to 3 cc. of blood or plasma) was oxidized with minimal quantities of sulfuric and nitric acids in a 50 cc. round bottomed flask. After diluting and neutralizing, the solution was made up to a definite volume and aliquots were taken for the determination of phosphate in the usual manner.

DISCUSSION.

Titratable Alkali.—According to Wilson and his collaborators (10), a condition of alkalosis exists after parathyroidectomy and is responsible for the tetany. It has since been shown (11) that their results are not good evidence for the existence of an alkalosis. That such alkalosis does not, in fact, exist has been shown by Hastings and Murray (12), Underhill and Nellans (13), and by Salvesen (14). Cruickshank (15) has observed a slight alkalosis, due to CO_2 deficit, before tetany appeared, after which a condition of alkalosis obtained. This would seem to indicate that hyperpnea is an earlier sign of parathyroid insufficiency than is tetany.

In these experiments, the titratable alkali of the blood was determined in one animal, just as tetany was developing on the day after the operation, and in another dog in active tetany. The calcium content of the serum of the first dog was 7.54 mg. per 100 cc. The values for titratable alkali obtained were 44.3, 35.3, and 27.4 cc. of 0.1 N alkali to methyl red, phenol red, and thymolphthalein, in the order given, in the first dog and 44.5, 37.6, and 25.8 cc. in the second. The averages of the results obtained upon normal dogs, under similar conditions in other experiments (4, 11), were 42.5, 37.1, and 28.9 cc. to the three indicators, in the same order. Evidently, there was no marked change after thyroparathyroidectomy.

Calcium.—The decrease in the calcium content of the blood or its plasma or serum after parathyroidectomy has been too well

established to need any additional proof. However, for the sake of completeness, our results will be presented in compact form. Tetany regularly developed when the calcium content of the serum fell to 7 mg. per 100 cc., but it frequently appeared when the calcium content was much greater than this. In fact, in two out of the fourteen experiments, it was higher than the

TABLE I.
Calcium Content of Dog Serum.

Dog No.	At operation.	Pretetanic.	In tetany.	After thyroparathyroidectomy but without tetany.	Remarks.
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
3 A			9.51		
5 A	13.7				
8 A			7.91		
3	11.0			9.96	6 days after operation.
4	10.9		6.91		
5	13.0		7.51		
6		8.21			
7	12.6		7.44		
8			7.54		
11			6.81		
12				11.1	2 days after operation.
12				14.0	7 days after operation.
14		9.33			
16			6.55		
17			8.60		
18			9.62		
21			6.95		
22		8.34	6.69		
23	12.7		8.07		
24		8.18	7.38		
25		9.25	7.37		
Average.	12.3±1.4	8.62±0.71	7.61±1.96		

highest observed in five successfully parathyroidectomized dogs on the day after the operation, but before tetany had yet developed. But, in no case in which serum was obtained from the same animal at both times was the concentration of calcium increased at the onset of tetany.

Sugar.—According to Underhill and Blatherwick (16), the concentration of sugar in the blood is regularly diminished after thyroparathyroidectomy, even before tetany appears. Hastings and Murray (12) were unable to confirm this and the few results reported by Salvesen (14) corroborate them in their claim that the concentration of sugar in the blood is very little changed. Under-

TABLE II.

Concentration of Sugar in the Blood of Dogs after Thyroparathyroidectomy.

Dog No.	Pretetanic.		In tetany.		After operation but without tetany.		Remarks.
	Benedict-Osterberg.	Folin-Wu.	Benedict-Osterberg.	Folin-Wu.	Benedict-Osterberg.	Folin-Wu.	
5				119			
6	154	151					
7			88	75			
11			137	119			
12					108	100	7 days after operation.
14	145	121	138	114			
16			140	133			
17			101	103			
18			182	90			
20						100	4 days after operation.
21			187	138			
22			127	111			
23			150	111			
24	160	137	139	140			
25	114		90	60			
Average...	114	136	136	109			

hill and Nellans (13) admit that the method of Forschbach and Severin employed by Underhill and Blatherwick in their second paper is open to criticism, but they claim to have observed the same hypoglycemia after thyroparathyroidectomy when the method of Folin and Wu was used. But reference to their figures shows that there was no decrease in the concentration of sugar until after tetany had appeared except in one experiment and

that, in this case, the decrease was not greater than the increase observed, at a similar time, in two other experiments. In other words, the change in the concentration of blood sugar was, at least until after the development of tetany, within the limits of error of their experiments. Whatever changes may have occurred after severe tetany had set in, may properly be ascribed to the effect of the tetany.

No normal controls were obtained in the course of the present investigation. Blood drawn under ether anesthesia would, of course, have been quite unsuitable for this purpose. But the values obtained after the operation and even after tetany had appeared are, with two exceptions, quite within the range generally regarded as normal, or even somewhat above this.

Nucleotide Nitrogen.—Because of the retention of phosphorus to be discussed presently, it seemed to be of interest to determine the nucleotide nitrogen in a few samples of blood. The values obtained in Dogs 3, 4, and 5, at operation, were 4.89, 4.38, and 1.23 mg. per 100 cc., respectively. The blood of Dogs 4 and 5, obtained when the animals were in tetany, contained 1.18 and 3.07 mg. per 100 cc., respectively.

Phosphorus.—In 1913, the author (17) reported that the blood and serum of parathyroidectomized dogs in tetany contained more "acid-soluble" phosphorus than did the blood or serum of normal dogs bled at the same interval after the last meal of an identical diet. Several years later, in connection with the experiments of Hastings and Murray (12), he analyzed the blood and plasma of dogs before and after thyroparathyroidectomy. The "acid-soluble" phosphorus was increased, but not nearly so much as in his earlier experiments. Rather slight increases in the inorganic phosphorus of the serum were also observed by Salvesen (14), but, in some instances, there was no increase at all. Gross and Underhill (18) found a marked increase in the total phosphorus of the blood after thyroparathyroidectomy in some dogs, but not in others. Their results cannot be properly interpreted because of the effect of possible changes in the lipoid phosphorus content and in the ratio of cells to plasma.

In the present series of experiment, the figures for the inorganic and the total "acid-soluble" phosphorus usually show only comparatively slight increases after removal of the thyroids and

TABLE III.

Concentration of Inorganic and Total "Acid-Soluble" Phosphorus in the Blood and in the Plasma of Dogs before and after Thyroparathyroidectomy.

Dog No.	Per 100 cc. whole blood.												Per 100 cc. plasma.							
	At operation.			Pretetanic.			In tetany.			No tetany.			At operation.		Pretetanic.		In tetany.		No tetany.	
	Cell volume.	Total.	Inorganic.	Cell volume.	Total.	Inorganic.	Cell volume.	Total.	Inorganic.	Cell volume.	Total.	Inorganic.	Total.	Inorganic.	Total.	Inorganic.	Total.	Inorganic.	Total.	Inorganic.
	per cent	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1 A								29.5	3.60								4.50	4.19		
3 A								34.0									13.14	12.00		
5 A											27.8	2.85*							3.44	2.48*
8 A								37.2	4.42									5.66		
1												2.09*							3.60	2.98*
2											25.0	3.68*							6.33	4.74*
3		26.0	2.50								24.1	2.37†	3.70	2.89					4.26	2.98†
4		27.2	3.36					29.0	4.55				6.24	5.13			7.44	6.59		
5		25.2	3.52					25.3	4.39				4.71	4.64			5.95	5.93		
6		30.6	3.32		20.1	3.33							4.90	5.10	5.28	4.94				
7		33.3	3.23					31.4	3.96				4.94	4.68			5.91	5.80		
8					22.1	4.31									7.00	6.73				
11	59.3	18.0	2.66				45.3	23.6	4.39				4.73	4.64			6.30	5.56		
12	48.1	24.5	4.67							42.6	23.7	4.16‡	6.00	6.12					7.54	7.58‡
										31.1	20.3	4.77§							6.31	6.28§
14	48.9	26.1	5.31	37.7	29.9	6.22	33.6	27.6	7.08				7.02	6.36	9.38	9.26	8.27	7.98		

15		20.5	4.28																	
16							46.9	23.5	3.54								5.54	3.77		
17	47.3	23.2	4.05				33.3	24.8	2.61				5.58	5.25			5.57	2.67		
18	56.1	28.4	4.53				51.3	26.9	4.70				7.09	5.92			6.73	6.34		
19	57.0	25.5	4.30										7.01	5.68						
20											24.7	5.06¶								
21							47.3	28.9	5.21								7.05	6.32		
22				32.7	21.5	6.93	29.6	26.8	7.63						8.74	7.20	9.21	8.07		
23	53.6	29.1	3.65				48.8	27.1	3.80				4.36	4.14			6.35	4.42		
24				49.1	29.4	4.45	30.0	27.5	5.38						7.62	5.71	7.28	6.48		
25				38.7	23.8	5.51	40.8	34.2	8.93						7.91	7.24	14.39	11.90		
Average	52.9	26.0	3.80	39.6	24.5	5.13	40.7	28.6	4.95		24.3	3.59	5.52	5.05	7.66	6.85	7.58	6.48	5.30	4.51
" of those for which cell volume is known	52.9	25.0	4.17	39.6	26.2	5.78	40.7	27.1	5.33		26.4‖	2.87‖	5.97	5.44	8.41	7.35	7.67	6.35	4.46‖	3.40‖

*No previous operation.

†6 days after thyroparathyroidectomy. No tetany. Serum calcium 9.96 mg. per 100 cc.

‡2 days after thyroparathyroidectomy. No tetany. Serum calcium 11.1 mg. per 100 cc.

§7 days after thyroparathyroidectomy. No tetany. Serum calcium 14.0 mg. per 100 cc.

¶4 days after thyroparathyroidectomy. No tetany.

‖ Average of figures obtained on animals not previously operated upon, Dogs 1, 2, and 8 A.

parathyroids. The possibility that differences in the analytical procedures may have been responsible for the differences in the results obtained in 1913 and in the present investigation did not suggest itself until the experiments had been concluded, but it is scarcely likely. Picric acid was used as the protein precipitant in 1913 (2), nitric acid in 1921 (11), and trichloroacetic acid in this series of experiments. Trichloroacetic acid was also used by Salvesen (14). There is no reason to believe that 1 per cent picric acid would liberate a form of phosphorus that would not be liberated by 5 per cent nitric acid or 4 per cent trichloroacetic acid.

At the risk of wearisome repetition, the author must again mention the remarkable decrease in the excretion of phosphorus in the urine after parathyroidectomy. There is no compensatory increase into the intestine. The amount retained varies between 20 and 60 mg. of phosphorus per kilo of body weight. There is no obvious relation between the amount retained and the severity of the symptoms or the rapidity with which they develop. If evenly distributed throughout the body, this retained phosphorus should produce an increase in the inorganic phosphorus of the blood greater than that observed in the experiments of Hastings and Murray (12), Salvesen (14), or in most of those now reported. However, the increases reported in 1913 (17) were of this order, or even greater, as are two of those observed in the present series (Dogs 3 A and 25).

As already stated, those of 1913 differed from all the subsequent experiments in that the control samples of blood were not taken from the same animals before the operation but from other animals bled under similar conditions. They also differed in that the analyses were made on blood obtained by complete exsanguination whereas, in all others, small samples of blood were used. But probably the most significant change was in the anesthetic used. In the earlier experiments, this (the bleeding) was accomplished under ether anesthesia and from a carotid artery. Later the dogs were bled from a femoral artery, using cocaine. Unfortunately, the note-books of 1911 to 1913 are no longer available and the published protocols do not indicate on which animals ether was used and on which, cocaine. But it appears to be significant that it was precisely in the earlier experiments of those reported in 1913 that the largest increases in phosphorus were observed.

The phosphorus retained after parathyroidectomy must be deposited in the tissues. Apparently, under certain conditions, such as ether anesthesia (possibly anoxemia accompanying the same), cerebral anemia, general anemia, or a combination of two or all of these, it may reappear in the circulation.[1]

The mechanism by which this retention of phosphorus may be related to the appearance of tetany will be discussed in a subsequent communication.

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[1] Since the above was written, Martland and Robison (Martland, M., and Robison, R., *Biochem. J.*, 1924, xviii, 765) have published the results of their experiments in which they found that the inorganic phosphate of the blood of normal rabbits was increased by ether anesthesia. A similar effect was observed in several dogs by Stehle and Bourne (Stehle, R. L., and Bourne, W., *J. Biol. Chem.*, 1924, lx, 17).

THE EFFECT OF ALCOHOLIC EXTRACT OF COMMERCIAL GRANULAR GLUCOSE ON URINARY REDUCING SUBSTANCE.*

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During the course of some experiments by us on commercial glucose, Folin and Berglund[1] published an article in which they maintained that the administration of C.P. glucose in amounts of as much as 200 gm. did not produce a glycosuria. We then determined to repeat our previous work, using both C.P. and commercial glucose, and to compare the behavior of the two. Finding a difference in the results with C.P. glucose, we tried to find out what the substance was in the commercial glucose which led to the increased excretion of reducing substance. In the first place, an alcoholic extract of the commercial glucose was prepared, and it was found that most of the coloring matter came down in the extract. This extract was then used in combination with the C.P. glucose. The effect produced by the mixture was compared with a control, the extract used alone, commercial glucose, and C.P. glucose. The work covered the determination of amounts of reducing substance in both blood and urine, and the chemical analysis of the sugars and extracts. The latter is still in progress.

EXPERIMENTAL.

Small weighed portions (about 50 gm.) of commercial granular glucose were ground into a fine powder and extracted repeatedly with small amounts

*Aided by a grant from the Gusta Morris Rothschild Fund of the Michael Reese Hospital.

[1] Folin, O., and Berglund, H., Some new observations and interpretations with reference to transportation, retention, and excretion of carbohydrates, *J. Biol. Chem.*, 1922, li, 213.

of 95 per cent redistilled alcohol until the extract, which was at first dark brown in color, became almost colorless. The combined portions of extract were then evaporated to a syrup and kept in glass-stoppered bottles. The concentration of the extract in terms of the original glucose was carefully noted. The sugar was taken in the form of lemonade and in each case 2 gm. per kilo of body weight were used. When the extract was used either alone or in combination with C.P. glucose the amount was equivalent to that obtained from 2 gm. per kilo of the commercial glucose. The C.P. glucose was Pfanstiehl's C.P. special. Six adult laboratory workers, five women and one man, served as subjects. Five sets of experiments were conducted, as follows: (1) 2 gm. per kilo of body weight of C.P. glucose; (2) 2 gm. per kilo of commercial glucose; (3) 2 gm. per kilo of C.P. glucose plus an equivalent amount of extract; (4) extract alone; (5) control. In the control experiment only water plus lemon was taken.

DISCUSSION.

From Fig. 1 it will be seen that the alcoholic extract when taken alone had little or no effect upon the amount of blood sugar. The very slight rise is accompanied by a speedy return to the basal value or even a further drop. Commercial and C.P. glucose, as well as C.P. glucose plus extract, gave rise to hyperglycemia, showing similar curves. In all cases the peak occurred at the end of the first 30 minute interval and the values returned to very near the basal at the end of the $2\frac{1}{2}$ hour period.

Strikingly different are the figures for urinary reducing substance, shown graphically in Fig. 2. All cases showed a rise at sometime during the 3 hours immediately following the ingestion of C.P. glucose. The peak, as in the case of previous work, did not follow a regular course, but when all figures were averaged it came at the end of 60 minutes, with a value of 21.0 mg.; or 80.4 per cent above the basal figure of 12.4 mg. At the end of 3 hours the average value of reducing substance excreted was still 24.6 per cent above the fasting value. The total amount excreted during the 3 hours averaged 115. 3 mg. The average 3 hour increase over the basal was 28.6 mg. The values which Folin and Berglund[1] gave were recomputed in order to compare their results with ours. All their cases showed a very slight increase in urinary sugar, with the exception of one case which rose to 15 mg. or 75 per cent above the basal value at the peak of the curve, coming after 2 hours. At the end of 4 hours this subject was still excreting 50 per cent more sugar than his fasting value.

The ingestion of commercial granular glucose was followed by a rise in urinary reducing substance which was always greater than that brought about by C.P. glucose. The peak with commercial glucose ran from 144.1 to 739.5 per cent above the fasting value and that for C.P. glucose from 4.3 to 196.3 per cent above the fasting value. The average peak for commercial glucose was 220.3 per cent above the fasting value, and occurred also after 60 minutes. The total amount excreted in 3 hours was 57 per cent higher than the corresponding amount for C.P. glucose in the

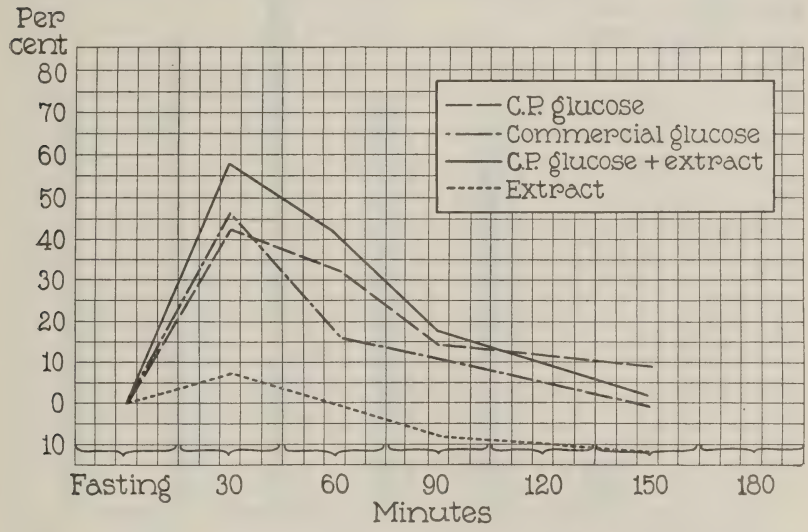

FIG. 1. Average percentage increase in reducing substance over the fasting values of blood after the ingestion of various grades of glucose.

same period. The average 3 hour increase was 101.7 mg. for commercial glucose and 28.6 mg. for C.P. glucose. The amount still being excreted at the end of the period was 108.1 per cent above the fasting value. Thus, no matter what method of comparison is adopted, the commercial glucose invariably leads to a markedly higher urinary sugar content than the C.P. sugar.

The ingestion of C.P. glucose plus extract gave rise to an entirely different situation. The extract contained only 10.4 per cent of sugar, but it was followed by an entirely disproportionate rise in urinary reducing substance. Here the peak values were likewise

scattered, but usually delayed, the average coming after 2 hours, and having a value 283.8 per cent higher than the basal. This is 29 per cent higher than the corresponding value for commercial glucose and 253 per cent higher than that for C.P. glucose alone.

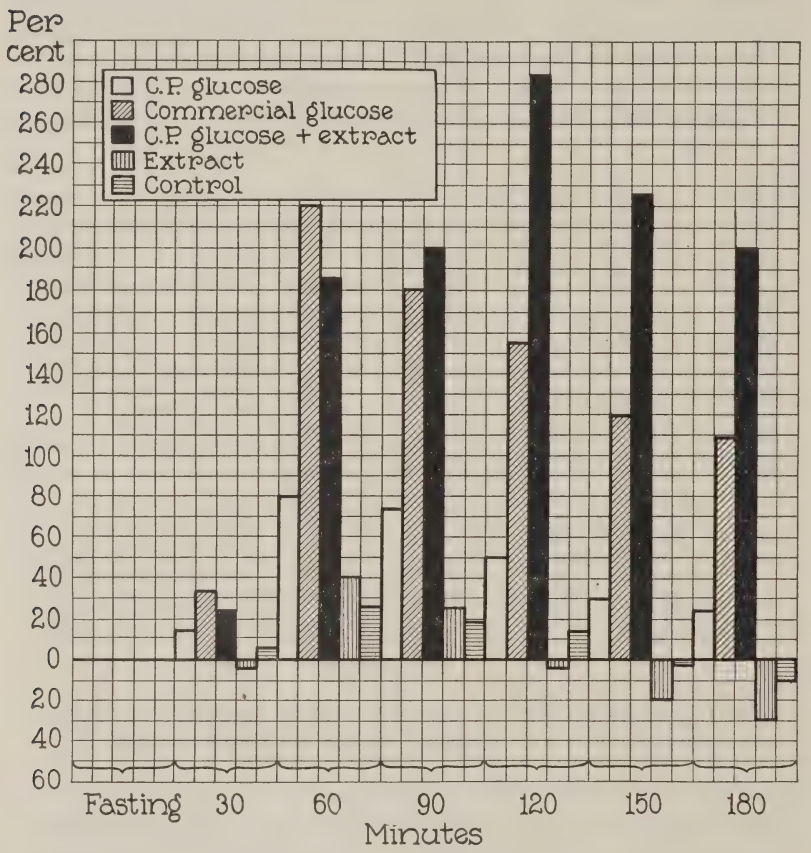

FIG. 2. Average percentage increase in reducing substance over the fasting values of urine after the ingestion of various grades of glucose.

The total amount of reducing substance in 3 hours ranged from 112.4 to 334.7 mg. with an average of 178.7 mg. The average 3 hour increase was 109.8 mg. These figures are closely similar to those of commercial glucose. At the end of the period, however, the average amount excreted was still 201.0 per cent above the

fasting value, which is nearly twice as high as the figure for commercial glucose in the corresponding interval, and somewhat more than eight times as high as the C.P. glucose.

The reducing substance excreted in 3 hours with C.P. glucose represents practically the total excess caused by the ingestion of 2 gm. per kilo of body weight, since the value returned almost to the basal in this time. With the commercial glucose, however, the excretion was still marked after 3 hours, so that an excess of this sugar probably continued to be excreted for some time afterward. When extract was added to the C.P. glucose the elimination of the excess of reducing substance was even more prolonged, and the 3 hour value represents considerably less of the total amount than in either of the two previous cases.

The ingestion of extract alone in 80 per cent of the cases was followed by a slight rise in the excretion of reducing substance. The peak occurred at the end of 1 hour in most cases, and the average was 41.3 per cent above the fasting value, but in every case it was followed by a decrease to below the fasting value. At the end of 3 hours the average excretion was 30 per cent below the basal. The average total excretion during the 3 hours was 90.0 mg. As a control experiment, lemon and water were used, and the effect was not essentially different from that of extract, although not so pronounced. The average peak, occurring also at 60 minutes, was 27 per cent above the fasting value, and at the end of 3 hours the reducing substance excreted was 10.1 per cent below the basal. The average total amount excreted during the 3 hours was 71.2 mg., slightly lower than with extract. Possibly Constam's[2] theory of a "rinsing out" process might account for some increased sugar excretion dependent upon the diuresis following a water intake.

The work done leads to the conclusion that there is some substance which may be extracted from commercial glucose with alcohol, which in itself has little effect on the amount of blood sugar and on the excretion of reducing substance, but which, in addition to C.P. glucose, invariably raises the amount of reducing substance in the urine. The blood sugar is only slightly affected. Although the extract contained 10.4 per cent of glucose, this

[2] Constam, G., Über den Einfluss peroraler Einnahme von Glucose auf Blutzucker und Glucurese beim Gesunden, *Biochem. Z.*, 1923, cxliii, 75.

amount added to the total amount of C.P. glucose ingested could not possibly account for the total increase in the excretion. Thus, in the case of the heaviest subject, weighing 67 kilos, the whole dose of extract taken contained only 2.2 gm. of glucose, which is 1.6 per cent of the total amount ingested, whereas the total increase in excretion of reducing substance was 284 per cent higher when the extract was added than when C.P. glucose was used alone. It is worthy of note that the extract added to the C.P. sugar had very little effect on the blood sugar curve, but very marked effect on the excretion of reducing substance even after the blood sugar had returned to the fasting value. This fact seems to indicate that the extract of commercial sugar acting in connection with C.P. glucose has a negative effect on the glycogen-forming process or the mechanism of tissue absorption. Benedict's[3] theory on the glycuresis following the ingestion of pure glucose is based on the assumption that the cells select from the mixture of α- and β- glucose more of one form than of the other, and the rest is eliminated. Possibly the extract has the power to change the chemical structure of the C.P. glucose ingested so that the less easily absorbed form predominates, and the elimination is increased. Further work on this subject is in progress in our laboratory.

SUMMARY.

1. A comparison was made of the reducing substance in blood and urine after the ingestion of 2 gm. per kilo of body weight of commercial granular glucose, C.P. glucose, C.P. glucose plus an alcoholic extract of commercial glucose, and extract alone.

2. Similar blood sugar curves occurred in the first three series. Extract alone gave a very slight rise in blood sugar.

3. All cases showed a rise in urinary reducing substance. The C.P. glucose showed the least rise and the quickest recovery; commercial glucose led to a much greater increase in urinary reducing substance. C.P. glucose plus extract gave a prolonged urinary excretion with the peak coming an hour later than in

[3] Benedict, S. R., and Osterberg, E., Sugar elimination after the subcutaneous injection of glucose in the dog. Including a discussion of the paper on observations on carbohydrates by Folin and Berglund, *J. Biol. Chem.*, 1923, lv, 769.

the other series. Extract alone, like the control, produced very little rise in urinary sugar.

4. The work leads to the conclusion that there is some substance which may be extracted from commercial glucose with alcohol, which in itself has little effect on the amount of blood sugar and on the excretion of reducing substance, but which, in addition to C.P. glucose, invariably raises the amount of reducing substance in the urine. The active principle in the extract seems to have a negative effect on the glycogen-forming process or the mechanism of tissue absorption.

A METHOD FOR THE QUANTITATIVE DETERMINATION OF MENTHOL GLYCURONIC ACID IN URINE.

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The study of glycuronic acid is important because it is concerned with an important detoxication mechanism of the body, and because of its possible relationship to carbohydrate metabolism. Our present knowledge of its synthesis in the body is meager and there is a distinct disagreement among the findings of the various investigators who have worked on the problem (1, 2). Much of this conflict in the experimental findings is undoubtedly due to the lack of a reliable quantitative method for glycuronic acid, or rather conjugated glycuronic acids, which can be applied to urine.

One of the oldest and most frequently used methods for determining conjugated glycuronic acids is based on optical rotation measurements. There are several serious disadvantages and objections to this method of analysis. In the first place, urine is an unsatisfactory solvent medium for polariscopic measurements, since its high concentration of salts and its variable hydrogen ion concentration introduce factors which can neither be controlled nor corrected satisfactorily. In the second place, the method fails completely unless the other optically active substances are removed. Glucose and other sugars may be removed by fermentation, but there is evidence that glycuronic acid is also slightly fermentable (3, 4). Finally, very few conjugated glycuronic acids have been prepared in a pure state and their optical rotation as well as the rotation of their salts studied with sufficient accuracy to be of value for quantitative analysis.

Another quantitative method proposed is based on the conversion of glycuronic acid to furfural when distilled with 12

per cent hydrochloric acid. This method, as outlined by Tollens (5), is purely empirical since the amount of furfural obtained is only one-third of the amount required by theory. The method further assumes that all conjugated glycuronic acids yield a definite amount of furfural, whereas Mann, Kruger, and Tollens (6) found that the amount varied with the individual acids.

As early as 1887 Thierfelder (7) found that a mol of glycuronic acid possessed the same reducing power on Fehling solution as a mol of glucose (1). No advantage of this property was taken until recently when Biberfeld (8) determined free glycuronic acid in urine by Bang's method and found that 1 mol of glycuronic acid was equivalent to 1 mol of glucose. The method, as employed by Biberfeld, is not directly applicable to conjugated glycuronic acids nor can it be used in the presence of other reducing substances, especially sugars. As already pointed out, sugars are especially objectionable since no common satisfactory method exists for their removal from urine without also destroying or removing some of the glycuronic acid.

Other means of determining glycuronic acid have been proposed from time to time. Thus, Neuberg and Neimann (9) suggested a determination of phenol glycuronic acid by simultaneous hydrolysis and oxidation of the compound with hydrochloric acid and bromine under pressure and determining the resulting saccharic acid as the silver salt. As the method does not give very accurate results and requires the use of sealed tubes, it has claimed little attention. Another method, devised by Vitali (10) for urochloralic acid, which requires a laborious reduction of trichloroethyl alcohol to alcohol (which is then determined), has met a similar fate.

Since none of the methods were satisfactory and since it was planned to investigate the relationship of glycuronic acid to glucose metabolism as studied by means of phlorhizin and pancreatic diabetes, efforts were made to develop a method which could be applied to urines containing sugar.

Attention was first directed to the furfural distillation method since it was hoped that by incorporating the modification of Pervier and Gortner (11) of steam distilling the furfural out of the reaction mixture, the great source of error, namely the destruction of furfural by prolonged contact with strong

acids, could be eliminated. It was found, however, that the conversion of glycuronic acid to furfural was so slow that a 0.2 gm. sample of menthol glycuronic acid yielded only a little more than one-half of the theoretical amount of furfural after 8 hours of continuous distillation and after 1,200 cc. of distillate had been collected.

The possibility of utilizing the reducing action of glycuronic acid and applying a quantitative sugar method for its determination was next investigated. This study lead to the development of a satisfactory method which is presented in detail.

Since it seemed almost impossible to find a general method which could be applied to all conjugated glycuronic acids, it seemed best to develop a method which was specific for one, but which, with slight modifications, could be extended to the determination of other conjugated glycuronic acids. Menthol glycuronic acid was chosen, for the acid can readily be isolated from urine and prepared pure so as to be suitable for a standard. It, furthermore, has the advantage of being easily soluble in ether and of being readily hydrolyzed by dilute acids. Menthol, itself, is a substance which is readily obtainable, and which is well suited for feeding experiments since animals tolerate it well and in considerable amounts.

The quantitative method developed for menthol glycuronic acid consists in the extraction of the compound from urine by means of ether, hydrolysis of the conjugated acid with a dilute mineral acid, and finally a quantitative determination of the liberated glycuronic acid by a sugar method. Since sugar and other common reducing substances are insoluble in ether the method is decidedly specific. By a preliminary ether extraction of the urine made alkaline with sodium carbonate, the specificity can be further enhanced since a number of substances such as aromatic hydroxy compounds, which might react with the sugar reagents, are thus removed, while the sodium salt of menthol glycuronic acid, which is insoluble in ether, stays behind. Then, on acidification the free acid is extracted. So far this preliminary extraction has not been found necessary. The hydrolysis of menthol glycuronic acid is brought about by boiling it with N hydrochloric acid under a reflux condenser.

The hydrolysate after neutralization and dilution to a definite volume is analyzed either by the Benedict or the Folin-Wu sugar method. Before Benedict's method could be applied to the dilute solutions used, the method had to be modified and rigorously standardized before trustworthy analytical results were obtainable. It was found that the oxidizing value of Benedict's solution is markedly influenced by the concentration of sodium carbonate, or, more exactly, by the hydroxyl ion concentration of the solution. To control this factor it was necessary, first, to add a constant weighed amount of anhydrous sodium carbonate, and secondly, to determine the oxidizing value of Benedict's solution for a series of dilutions of varying concentrations such as would be obtained by titrating with solutions of different strengths of glycuronic acid. From the data obtained Chart 1 was constructed from which the oxidizing value, *i.e.* the glycuronic acid equivalent per cc. of Bendict's solution, can be read directly. Although Benedict's solution when carefully prepared according to directions and with pure chemicals has a constant and definite strength, it seemed desirable to be able to standardize the solution with glucose. It was found that while 1 mol of glycuronic acid has approximately the same reducing power as 1 mol of glucose, a slight difference exists since the concentration of sodium carbonate influences the oxidation of glycuronic acid slightly more than it does the oxidation of glucose. A 0.125 per cent solution of glucose is recommended for the standardization of Benedict's solution. Curiously, while 1 mol of glycuronic acid is equivalent to 1 mol of glucose in its power to reduce Benedict's solution, 1.5 mols of glycuronic acid are necessary for a similar action on the reagent of Folin and Wu.

By using the Benedict as a macro, and the Folin-Wu as a micro method, it is possible to get good results over a wide range of concentrations of conjugated glycuronic acids in urine. As the size of the sample can also be varied, the method is applicable to the whole range of concentrations from the highest possible to a few milligrams per 100 cc. The method, furthermore, possesses an accuracy equal to that of the quantitative sugar methods with the added advantage that it is more specific, which is of great importance when analyzing urine containing more than

one reducing substance. While the method has been applied only to menthol glycuronic acid, there is no reason why it cannot be extended to the analysis of other glycuronic acids since a great many of them are soluble in ether and are hydrolyzed by dilute acids. Preliminary work on thymol glycuronic acid seems to indicate that the time of hydrolysis may have to be lengthened, but fortunately glycuronic acid is not destroyed by prolonged boiling with dilute acid. The analysis of phenol glycuronic acid and others of physiological importance will be studied in detail and reported later.

EXPERIMENTAL.

Preparation of Menthol Glycuronic Acid.—This compound is best isolated from urine by Bang's method (12) which consists in the precipitation of the ammonium salt of menthol glycuronic acid by half saturating the urine with ammonium sulfate. The complete procedure for obtaining the compound was as follows: 2 gm. of menthol were mixed with hot water and allowed to stand until it was liquefied when it was emulsified by shaking and administered to the rabbit through a stomach tube. The urine excreted during the 24 hours following the feeding was collected, made alkaline with ammonium hydroxide, heated to boiling, half saturated with solid ammonium sulfate, and filtered hot. On cooling the ammonium salt separated out almost completely, less than 0.4 gm. per 100 cc. remaining in solution. The product obtained was generally colorless and free from gummy impurities, and a second precipitation with ammonium sulfate rendered it pure white. The free acid was prepared by dissolving the ammonium salt in the minimum amount of hot water and adding a calculated amount of N hydrochloric acid to liberate completely the free acid. On cooling the acid crystallized out and one additional crystallization from hot water was sufficient to render the compound analytically pure. Unfortunately, the acid contains a variable amount of water. Fromm and Clemens (13) state that it has 1.5 molecules of water of crystallization, but repeated analyses showed a content somewhat midway between that calculated for 1.5 and 2 molecules. Over fused calcium chloride or sulfuric acid it lost water rapidly until its water content corresponded to 1 molecule of water of

crystallization. After this the rate of loss was slow, indicating that the vapor tension of the monohydrate is slightly higher than that of the drying agent. Because of this variable water content, the samples used for analysis were dried to constant weight at 100° under vacuum and all calculations based on anhydrous menthol glycuronic acid.

TABLE I.

Concentration of menthol glycuronic acid.	Concentration of glycuronic acid.	Final concentration of sodium carbonate.	Titration.	Glycuronic acid equivalent per cc. of Benedict's solution.
per cent	*per cent*	*gm. per 100 cc.*	*cc.*	*mg.*
0.1834	0.107	9.26	22.4	2.42
0.2000	0.117	9.94	20.0	2.37
0.2292	0.134	10.99	17.3	2.31
0.2750	0.161	12.55	13.9	2.23

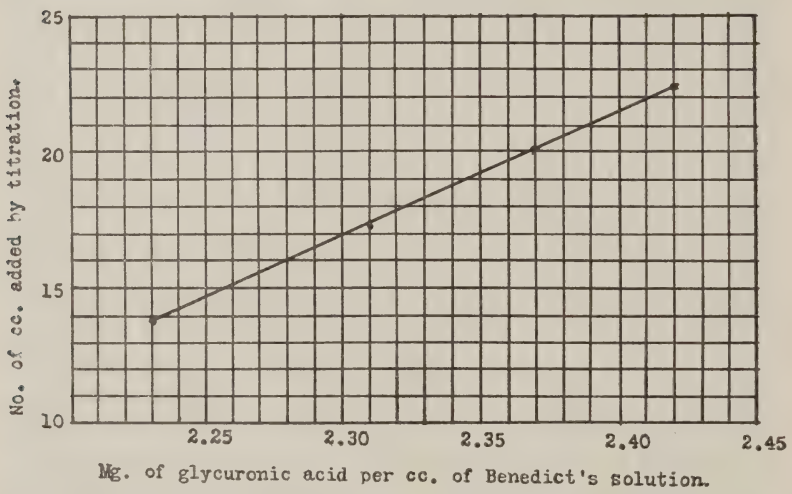

CHART 1.

Standardization of Benedict's Solution.—A weighed sample (180 to 275 mg.) of anhydrous menthol glycuronic acid was refluxed with 10 cc. of N hydrochloric acid for 15 minutes to complete hydrolysis. On cooling the solution was neutralized with N sodium hydroxide and diluted in a volumetric flask to such a volume that the glycuronic acid content was 0.10 to

0.015 per cent. With such dilute solutions, Benedict's reagent had to be carefully standardized for varying concentrations of sodium carbonate. As the concentration depends both on the amount initially present (the amount in the reagent itself and the amount added); and on the final dilution, *i.e.* the volume of the reagent plus the volume added by titration; it was necessary, first to add a constant weighed amount of anhydrous sodium carbonate, and secondly, to determine the oxidizing power (the glycuronic acid equivalent) of Benedict's solution for the range of dilutions produced by adding from 10 to 25 cc. in the titration. The results recorded in Table I were obtained by using 10 cc. of Benedict's solution and 2 gm. of anhydrous sodium carbonate. The titrations were carried out in a 100 cc. Erlenmeyer flask instead of an open porcelain dish to minimize both the loss of water by evaporation and the reoxidation of the reduced copper.

Three different samples of Benedict's solution were found identical in strength. Besides having been standardized with menthol glycuronic acid, they were also standardized with a 0.125 per cent solution of glucose. In each titration 2 gm. of anhydrous sodium carbonate were added.

Found:

10 cc. of Benedict's solution required 16.6 cc. of a 0.125 per cent glucose solution.

10 cc. of Benedict's solution required 16.6 cc. of a 0.139 per cent glycuronic acid solution.

1 cc. of Benedict's solution = 2.08 mg. glucose or 2.30 mg. glycuronic acid.

Ratio: Glycuronic acid: glucose = 1.105.

Calculated:

Glycuronic acid: glucose = 1.077 (assuming 1 mol of glycuronic acid is equivalent to 1 mol of glucose).

Standardization of the Folin-Wu Reagent.—A sample of anhydrous menthol glycuronic acid was hydrolyzed as described above, neutralized, and diluted to such a volume that the solution contained approximately 250 mg. of glycuronic acid per 100 cc. The reducing value of this solution was determined in the precise manner as that of an unknown sugar solution.

45.86 mg. of menthol glycuronic acid were dissolved in 100 cc. of water.

1 cc. contained 0.459 mg. menthol glycuronic acid, or 0.267 mg. glycuronic acid.

Found:

The reducing value of 1 cc. = 0.177 mg. glucose, therefore,
" " " " 0.267 mg. glycuronic acid = 0.177 mg. glucose
and
the reducing value of 1.51 mg. glycuronic acid = 1.0 mg. glucose.

Extraction of Menthol Glycuronic Acid.—Menthol glycuronic acid was quantitatively removed from urine with ether, using a continuous extractor of the type designed by Clausen (14). The apparatus was, however, modified so as to be adapted to the analysis of 10 cc. samples. The lower or constricted portion of the extraction tube was 180 mm. long and had an internal diameter of 14 mm. The lower end of the inner tube was a small perforated bulb such as is commonly employed in aeration with the exception that the perforations came out obliquely upwards instead of laterally. A well insulated air bath supplied with a 75 watt nitrogen-filled lamp produced a well controlled and steady flow of ether through the extractor. With this apparatus complete extraction was effected in $2\frac{1}{2}$ hours. It is advisable, however, when using a new extractor to redetermine the time necessary for complete extraction, and for very important analyses to run the second sample a half an hour longer to determine whether the extraction of the first sample was complete. Since it is common practice in extractions to saturate the solution with ammonium sulfate, the procedure was tried, but it hindered rather than aided the extraction.

Procedure of Analysis.—A 10 cc. sample of filtered urine (5 cc. if the concentration of menthol glycuronic acid was known to be high) was transferred to the extraction tube and acidified with 1 cc. of 20 per cent sulfuric acid. 50 cc. of ether were placed in the boiling flask, and the extraction was carried out for $2\frac{1}{2}$ hours. The ether extract was then quantitatively removed to a 100 cc. Erlenmeyer flask, the ether boiled off, and the residue hydrolyzed by refluxing it for 15 minutes with 10 cc. of N hydrochloric acid. On cooling the solution was made neutral to litmus with N sodium hydroxide. If the approximate concentration of menthol glycuronic acid was not known, the solution was diluted to a small volume (25 cc. or even less) and the approximate concentration of glycuronic acid determined, using an aliquot. If the concentration was found to be 0.1 per cent or higher

the Benedict method was employed, while for lower concentrations the Folin-Wu method was used. For the Benedict method the solution was diluted in a volumetric flask to such a volume that the concentration of glycuronic acid was 0.10 to 0.15 per cent. It was filtered to remove undissolved menthol which has a tendency to adhere to glass and thus cause imperfect drainage of the burette. 10 cc. of Benedict's reagent and 2 gm. of anhydrous sodium carbonate (weighed to within 0.05 gm.) were placed in a 100 cc. Erlenmeyer flask and heated to boiling. After the sodium carbonate had dissolved, the glycuronic acid solution was added from a burette at such a rate that constant boiling of the reagent could be maintained. Near the completion of the titration the solution was added drop by drop, allowing at least 10 seconds to elapse between each addition. The complete disappearance of the blue color (green, if the solution was yellow) was taken as the end-point. With a little practice the end-point of duplicate samples could easily be checked to 0.1 cc.

Calculation (Benedict's method).

$$10 \times \frac{\text{Glycuronic acid equivalent per cc. of Benedict's solution*}}{} \times \frac{\text{Volume after dilution}}{\text{Titration in cc.}} \times \frac{\text{Total volume of urine}}{\text{Volume of urine taken}} = \text{Gm. glycuronic acid in total volume of urine}$$

The Folin-Wu method was used without modification. The glycuronic acid solution was diluted so that its concentration was between 0.01 and 0.015 per cent. The reducing power in terms of glucose of this solution was determined, and the result multiplied by the factor, 1.51, gave the glycuronic acid content.

A Typical Analysis.—This analysis was made on a 24 hour specimen of urine collected after a rabbit had been fed 3.5 gm. of menthol.

Volume of urine ..... 122 cc.
" " samples extracted ..... 5.0 "

Analysis by the Benedict Method.

Volume of hydrolyzed extract ..... 50 cc.
Titration. I. 19.2 cc.
II. 19.4 "

* Obtained from Chart 1.

Found. I. 1.49 gm. glycuronic acid or 2.56 gm. menthol glycuronic acid.
II. 1.48 gm. glycuronic acid or 2.54 gm. menthol glycuronic acid.

Analysis by the Folin-Wu Method.

Volume of hydrolyzed extract ..... 250 cc.
Strength of glucose standard ..... 0.15 mg. per cc.
Reading of standard. I. 20 mm. Reading of sample. I. 18.0 mm.
II. 20 " II. 18.3 "
Found. I. 1.54 gm. glycuronic acid or 2.62 gm. menthol glycuronic acid.
II. 1.51 gm. glycuronic acid or 2.58 gm. menthol glycuronic acid.

By Direct Isolation of the Ammonium Salt.

Ammonium menthol glycuronate isolated from 105 cc. of urine ..... 1.75 gm.
Ammonium menthol glycuronate calculated for 122 cc. of urine ..... 2.04 "
Ammonium menthol glycuronate remaining in solution .. 0.45 "
Total ..... 2.49 "
Calculated as menthol glycuronic acid ..... 2.36 "

TABLE II.

Sample No.	Volume of urine.	Menthol glycuronic acid added.	Menthol glycuronic acid recovered.	Method of analysis.
	cc.	*mg.*	*mg.*	
1*	10	114.5	114.0	Benedict.
2†	10	91.7	91.5	"
3	10	91.7	91.5	"
4	10	36.7	34.6	Folin-Wu.
5	10	45.9	47.0	"
6	10	45.9	46.1	"

* Sample 1 also contained 1 gm. of glucose.
† The extract of Sample 2 was hydrolyzed 2 hours.

SUMMARY.

The quantitative methods for free and conjugated glycuronic acids are discussed and their defects pointed out.

A new quantitative method is proposed for menthol glycuronic acid, which depends on the extraction of the compound from urine with ether, with subsequent hydrolysis and the determination of the liberated glycuronic acid by means of either the

Benedict or the Folin-Wu sugar method. Modifications of the Benedict method which are necessary to make the method applicable to dilute reducing solutions are described.

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Addendum.—After this paper had been sent to press, an article by Csonka (Csonka, F. A., *J. Biol. Chem.*, 1924, lx, 545) appeared in which a determination for benzoyl glycuronic acid is outlined. The method is similar to the one presented above in that the benzoyl glycuronic acid is hydrolyzed by means of dilute acid and the liberated glycuronic acid determined by Benedict's quantitative sugar method. It differs, however, in the isolation of the conjugated acid from urine. The compound is precipitated with basic lead acetate, and the lead subsequently removed by means of hydrogen sulfide.

In the development of the quantitative method for menthol glycuronic acid, its removal from urine by means of basic lead acetate was considered but abandoned in favor of the extraction method; because the latter procedure is less cumbersome, is more applicable to small volumes of urine and small amounts of glycuronic acid, and is more specific since basic lead acetate will also precipitate other reducing substances, especially sugars. Since it is very likely that benzoyl glycuronic acid is soluble in ether, it ought to be readily and accurately determined with the method outlined for menthol glycuronic acid.

THE SYNTHESIS OF MENTHOL GLYCURONIC ACID IN THE RABBIT.

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The lack of a trustworthy analytical method for conjugated glycuronic acids heretofore has left many problems concerning their synthesis in the body unsolved. Before attempting, however, any specialized phase of the problem, it seemed necessary to study the excretion of conjugated glycuronic acids following the feeding of a substance like menthol from the point of view of: the effect of dosage; the rate of excretion; and the effect of prolonged feeding.

Fromm and Clemens (1) state that a strong rabbit can tolerate as much as 5 gm. of menthol, and Biberfeld (2) reports that a rabbit can be fed 2 gm. daily for a period of over a month provided greens are included in the diet. No reference was found concerning the relative amount of menthol conjugated. Von Fenyvessy (3) from polariscopic measurement determined that 2.6 to 2.8 gm. of camphorol glycuronic acid were produced following the administration of 2 gm. of camphor.

In the experiments carried out, it was found that the rabbit conjugated somewhat less than one-half of the menthol fed up to a certain amount after which the amount conjugated decreased as the dose was increased, but that, nevertheless, the amount conjugated was high even for the lethal dose provided the animal lived 10 hours after the feeding. There seemed to be only slight variations between rabbits as to the amount of menthol they could conjugate. Somewhat less than 3 gm. of menthol glycuronic acid were the maximum amount that a 2 kilo rabbit could excrete, and this was obtained by feeding about 3.5 gm. of menthol. No information was obtained concerning the elimina-

tion of the unconjugated menthol. The lethal dose was found to be about 4 to 5 gm., but frequently the animal showed signs of distress when fed only 3.5 gm., especially when given in alcohol as recommended by Neuberg and Lachmann (4). Temporary paralysis of the hind legs was a common symptom observed in menthol poisoning, and often there was a loss of appetite.

If it be assumed that glucuronic acid is the product of a detoxication mechanism, it is reasonable to suppose that the conjugation takes place soon after the substance passes through the intestinal wall and probably even before, so that the rate of excretion is dependent on the speed of absorption of the active compound from the intestines. Menthol glycuronic acid appeared in the urine in less than an hour after feeding menthol, and 90 per cent of the conjugated acid was found to be excreted in 6 hours when 2 gm. of menthol were fed. Even when larger doses were given, over 90 per cent of the total amount excreted appeared in the urine during the first 24 hours.

A study of the effects of prolonged feeding seemed to be of importance. Biberfeld (2) reported that after feeding a rabbit 2 gm. of menthol daily, it excreted only 30 per cent as much menthol glycuronic acid on the 10th day as it did on the 1st, and that only a trace was found in the urine after feeding menthol for a month. If Biberfeld's findings be correct, the results of many experiments in which glycuronic acid-producing substances were fed over long periods of time would be vitiated.

Biberfeld's results could not be duplicated, however. While the excretion of menthol glycuronic acid varied somewhat from day to day, no marked increase or decrease was observed. One rabbit which was fed 2 gm. of menthol daily for 24 days had a slightly higher output of the conjugated acid at the end, and appeared normal and healthy at the completion of the experiment. It is difficult to explain Biberfeld's results. Of course the use of an untrustworthy analytical method might easily lead to erroneous conclusions and a faulty or incomplete diet might cause abnormal results.

The study of the synthesis of conjugated glycuronic acids is being continued and an investigation of the formation of glycuronic acid in experimental diabetes is now under way.

TABLE I.

Menthol fed.	Rabbit I.		Rabbit II.	
	Menthol glycuronic acid excreted.	Menthol conjugated.	Menthol glycuronic acid excreted.	Menthol conjugated.
gm.	*gm.*	*per cent*	*gm.*	*per cent*
0.5	0.51	48.0		
1.0	0.88	41.3	0.98	46.0
2.0	1.80	42.3	2.02	47.5
2.5	2.02	37.9		
3.0	2.36	37.1	2.60	40.7
3.5	2.74	36.6	2.60	34.9
4.0			2.22	26.1
4.5			1.82	19.0
5.0			1.80 (approximate).	16.9

TABLE II.

Rate of Excretion.

Rabbit III.

Time.	Menthol fed.	Volume of urine.	Menthol glycuronic acid excreted.	Percentage of total.
1924	*gm.*	*cc.*	*gm.*	*per cent*
May 27, 7.25 a.m.	2.0			
" 27, 10.25 "		41	0.71	37.2
" 27, 1.25 p.m.		40	0.81	24.4
" 27, 4.25 "		78	0.27	14.1
" 27, 7.25 "		127	0.08	4.2
" 28, 9.00 a.m.		100	0.04	2.1

TABLE III.

Rate of Excretion.

Rabbit IV.

Time.	Menthol fed.	Menthol glycuronic acid excreted.	Percentage of total.
1924	*gm.*	*gm.*	*per cent*
May 17, 9.00 a.m.	3.5		
" 18, 9.00 "		2.74	93.83
" 19, 9.00 "		0.18	6.17

EXPERIMENTAL.

Rabbits were used in all the experiments. Menthol emulsified by shaking with hot water was fed to the animals through a stomach tube. The urine was collected for 24 hours after feeding except when large doses of menthol were fed when a 36 hour specimen was collected instead. The animals were main-

TABLE IV.

Effect of Prolonged Feeding of Menthol.

Rabbit V.		Rabbit III.		Rabbit I.	
Date.	Menthol glycuronic acid excreted.	Date.	Menthol glycuronic acid excreted.	Date.	Menthol glycuronic acid excreted.
1924	*gm.*	*1924*	*gm.*	*1924*	*gm.*
Apr. 9	1.26	May 6	1.85	May 6	1.85
" 10	1.13	" 7	1.66	" 7	1.60
" 11	1.39	" 8	1.50	" 8	1.90
" 12	1.03	" 9	1.33	" 9	1.82
" 14	1.39	" 10	1.82	" 10	1.66
" 15	1.17	" 11	1.49	" 11	1.82
" 16	1.08	" 12	1.35	" 12	1.55
" 17	1.54	" 13	1.52	" 13	1.77
" 18	1.32	" 14	1.43	" 14	1.95
" 19	1.17	" 15	1.44	" 15	1.85
" 20	1.46	" 16	1.35	" 16	1.62
" 21	1.32			" 17	1.70
" 27	1.37			" 18	1.64
" 28	1.40			" 19	1.75
" 29	1.50				
" 30	1.53				
May 1	1.57				
" 2	1.48				

Each rabbit received 2 gm. of menthol daily.

tained on a diet of oats and greens and an effort was made to keep their weights as constant as possible. All analytical data were obtained by the method described in the previous paper.

Effect of Dosage.

In the experiments recorded in Table I menthol was fed every other day in order to give the animals an opportunity to re-

cover and to rest, and also to have complete elimination of menthol before administering more. Rabbit II died 26 hours after receiving 5 gm. of menthol.

SUMMARY.

The synthesis of menthol glycuronic acid in the rabbit was studied. The response to dosage, the rate of excretion, and the effect of prolonged feeding of menthol were determined.

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A CONTRIBUTION TO THE CHEMISTRY OF GRAPE PIGMENTS.

III. CONCERNING THE ANTHOCYANS IN SEIBEL GRAPES.

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INTRODUCTION.

The anthocyan pigments occurring in dark blue American grapes such as the Norton and Concord[1] and the Clinton[2] have been found to be identical in composition and reactions. They were monoglucosides that formed oxonium salts with acids. Of these salts the picrates were found to crystallize readily; they were sparingly soluble in water and offered a convenient method of isolating the pigments. The glucosides were hydrolyzed easily when boiled with dilute hydrochloric acid, yielding 1 molecule each of glucose and anthocyanidin. The anthocyanidin consisted largely of a monomethyl ether of delphinidin, but the values for methoxyl were high, indicating that some dimethyl ether of delphinidin was also present. Our results were in substantial agreement with those of Willstätter and Zollinger[3] with respect to the anthocyan isolated from *Vitis riparia*.

The dark blue European grapes, *Vitis vinifera*, contain according to the investigation of Willstätter and Zollinger[4] the monoglucoside, oenin, which on hydrolysis yields 1 molecule each of glucose and the sugar-free pigment oenidin. The above authors have shown that oenidin consists largely of a dimethyl ether of delphinidin and that it also probably contains some monomethyl ether of delphinidin.

[1] Anderson, R. J., *J. Biol. Chem.*, 1923, lvii, 795.
[2] Anderson, R. J., and Nabenhauer, F. P., *J. Biol. Chem.*, 1924, lxi, 97.
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[4] Willstätter, R., and Zollinger, E. H., *Ann. Chem.*, 1915, cdviii, 83.

A study of the anthocyans occurring in hybrid grapes obtained by crossing European with American varieties might yield some information regarding the inheritance and transmission of pigments. It appeared of interest, therefore, to investigate the anthocyans contained in the dark blue Seibel grapes.[5] The grapes used consisted mostly of Seibel No. 78 and No. 2044 and represent, therefore, *Vitis æstivalis*, *Vitis rupestris*, and *Vitis vinifera*.[6]

The pigment was isolated as the picrate in the manner described in an earlier paper[2] and the picrate was converted into the chloride by the method of Willstätter and Zollinger.[4] The anthocyanin chloride crystallized in prisms from a mixture of methyl and ethyl alcohol containing dilute hydrochloric acid in the same manner as has been described for oenin chloride by Willstätter and Zollinger.[4] The substance was found to be a monoglucoside which gave, when hydrolyzed by boiling with 20 per cent hydrochloric acid, 1 molecule each of glucose and the sugar-free pigment, anthocyanidin chloride.

The crystalline anthocyanin chloride corresponded to the formula $C_{23}H_{25}O_{12}Cl + 3\ H_2O$. It gave no color reaction with ferric chloride, differing in this respect from the pigments isolated from American grapes which give an intense blue and purple coloration with this reagent. Judging by crystal form, composition, and reactions, the substance is identical with oenin chloride.

The anthocyanidin chloride was obtained in beautiful prisms on hydrolyzing the glucoside. Results were obtained on analysis that agreed with the formula $C_{17}H_{15}O_7Cl + 1.5\ H_2O$. In the Zeisel determinations the results showed that the substance consisted largely of a dimethyl ether of delphinidin, but the values for methoxyl were low indicating the presence of some monomethyl ether of delphinidin. Crystals of delphinidin iodide separated when the reaction mixture from the Zeisel determinations were allowed to cool.

The composition of the anthocyanidin chloride indicates that it is essentially similar to or identical with the oenidin chloride

[5] The grapes were grown in the New York Agricultural Experiment Station vineyard at Fredonia, N. Y., and we are indebted to Mr. F. E. Gladwin for the material used in this investigation.

[6] Viala, P., and Vermorel, V., Traité général de viticulture. Ampélographie, Paris, 1909, vii, 177–182.

obtained by Willstätter and Zollinger from *Vitis vinifera*. In this connection it is interesting to note in a cross between American varieties and *Vitis vinifera* that the pigment of the European grape is inherited by the hybrid.

EXPERIMENTAL PART.

Isolation of the Pigment as Picrate.

The skins were separated by hand and the juice was removed as much as possible by pressing in a hydraulic press. The pressed skins, 2.7 kilos, were digested in 2 liters of 0.5 per cent hydrochloric acid for 24 hours. The material was then rubbed to a pulp in a mortar and the liquid was expressed with a hydraulic press. The press-cake was stirred up with 1 liter of 0.5 per cent hydrochloric acid and again pressed. The extract, after it had been filtered through a layer of paper pulp, was perfectly clear and was of an intensely dark red color. It measured about 4 liters. It was warmed on the water bath to 45° and 40 gm. of finely powdered picric acid were added. The picric acid dissolved in the warm solution and the picrate began to separate in amorphous flakes. The precipitate was dissolved by warming the solution and on cooling the picrate crystallized in fine red needles or prisms. After the mixture had stood overnight the crystals were collected on a Büchner funnel and dried in a vacuum desiccator over sulfuric acid and potassium hydroxide. The dried substance formed a dark red powder that weighed 17.3 gm.

Preparation of Anthocyanin Chloride.

The crude picrate was powdered and dissolved in 250 cc. of methyl alcohol and 70 cc. of methyl alcohol, containing 19 per cent of dry hydrochloric acid, were added. A small amount of insoluble matter was filtered off and washed with a few cubic centimeters of methyl alcohol. The methyl alcoholic solution was of brilliant red color. The anthocyanin chloride was precipitated by adding 2,400 cc. of anhydrous ether. The amorphous precipitate was filtered and washed with anhydrous ether until free from picric acid. After it had been dried in a vacuum desiccator over sulfuric acid and potassium hydroxide it formed a dull dark red powder that weighed 12 gm.

Crystallization of Anthocyanin Chloride.

The amorphous product mentioned above was crystallized by the method of Willstätter and Zollinger[4] as follows: 3 gm. of the crude glucoside were dissolved in 10 cc. of methyl alcohol by gently warming on the water bath. The solution was filtered to remove some filter paper fibers and the flask and filter were washed with 2.5 cc. of methyl alcohol. The intensely dark red solution was mixed with 10 cc. of 5 N hydrochloric acid and the solution was allowed to stand in a loosely covered dish at room temperature. Since nothing had separated at the end of 24 hours 20 cc. of ethyl alcohol were added and the solution was allowed to stand as before. Crystallization began after the solution had stood for a few hours and after 2 days the crystals were filtered, washed with dilute hydrochloric acid, and allowed to dry in the air. It weighed 2 gm. When examined under the microscope it was seen to consist of long prismatic crystals; one end was flat and the other was pointed as a wedge. The crystals appeared to be perfectly homogeneous and there were no amorphous particles present. Individual crystals appeared to be nearly black in color. The dry substance showed a bronze luster in reflected light. Heated in a capillary tube the substance sintered at about 158° and it fused at about 160°. In a sealed capillary tube it fused at about 162°.

The substance was analyzed after it had been dried in a vacuum at 105° over phosphorus pentoxide. The loss in weight on drying was 8.74 per cent which corresponds to 3 molecules of water of crystallization. Calculated for 3 H_2O: H_2O 9.27 per cent.

Analyses. 0.1700 gm. substance: 0.0754 gm. H_2O and 0.3237 gm. CO_2. 0.2434 gm. substance: 0.0686 gm. AgCl.

Calculated for $C_{23}H_{25}O_{12}Cl$ (528.5). C 52.22, H 4.73, Cl 6.71 per cent.
Found. C 51.93, H 4.96, Cl 6.97 per cent.

Reactions of Anthocyanin Chloride.

A dilute solution of the glucoside in ethyl alcohol gives with a very dilute aqueous solution of ferric chloride a very faint reaction; the original purplish red color deepens, but no intense purple or blue coloration is developed as in the case of the glucoside obtained from Norton, Concord, or Clinton grapes. In aqueous solution the addition of ferric chloride gives no immediate

appreciable change in color, but after standing for a short time the red color fades, leaving a faint straw-colored solution.

In aqueous solution the following reactions were observed. The pigment is completely precipitated even from very dilute solutions with lead acetate as a blue flocculent precipitate. Copper sulfate gives a deep purple precipitate. Ammonium hydroxide gives a blue color. Sodium hydroxide and carbonate give green colors. The immediate addition of acid restores the red color, but if the alkaline solution has stood for some time the original color is not restored by acid. After sodium hydroxide or carbonate have been added alternately with hydrochloric acid four or five times the color produced by the alkali is pure blue. Sodium acetate gives a bluish green color.

When the aqueous solution, acidified with hydrochloric acid, is extracted with amyl alcohol the latter takes up some of the coloring matter assuming a purplish red color. This color cannot be removed completely on shaking the amyl alcohol with water acidified with hydrochloric acid, indicating the presence of a small amount of free anthocyanidin.

Hydrolysis of the Glucoside.

Isolation of Anthocyanidin Chloride.

The anthocyanin chloride, 3 gm., was dissolved in 60 cc. of water by gently warming on the water bath and 42 cc. of concentrated hydrochloric acid were added. The solution was heated to boiling and it was boiled for 5 minutes. Crystals began to separate after the solution had boiled for $3\frac{1}{2}$ minutes. After the solution had cooled and had stood in the ice box for several hours, the crystals were filtered, washed with dilute hydrochloric acid, and allowed to dry in the air. The substance weighed 1.6 gm. The macroscopic appearance of the crystals was nearly black. Examined under the microscope they were seen to consist of well formed prismatic crystals and there were no amorphous particles; single crystals appeared to be of pale brownish red color. Heated in a capillary tube the substance did not melt or appear to undergo any change when heated to 260°.

The crystals appeared to be perfectly pure and homogeneous and they were analyzed after drying at 105° in a vacuum over phos-

phorus pentoxide. The loss in weight on drying was 6.44 and 6.70 per cent, corresponding to 1.5 molecules of water of crystallization. Calculated for 1.5 H_2O: H_2O 6.86 per cent.

Analyses. 0.1220 gm. substance: 0.0461 gm. H_2O and 0.2470 gm. CO_2. 0.1307 gm. substance: 0.0520 gm. AgCl.

Calculated for $C_{17}H_{15}O_7Cl$ (366.5). C 55.66, H 4.09, Cl 9.68 per cent.

Found. C 55.21, H 4.22, Cl 9.84 per cent.

Reactions of Anthocyanidin Chloride.

The aqueous solution of the pigment gave the following reactions. Lead acetate precipitates the pigment completely even from very dilute solutions as a purplish blue precipitate. Copper sulfate gives no precipitate in dilute solutions, but the color changes from red to dull brownish red and later to purplish. On addition of ferric chloride the color deepens momentarily, but soon fades, leaving a nearly colorless solution. Sodium acetate precipitates the pigment as a reddish brown flocculent precipitate. Ammonium and sodium hydroxides give fine blue colors that fade after a few minutes to yellowish green. When the blue-colored solutions are acidified the original color is restored, but addition of acid to the faded solutions does not restore the red color even when heated to boiling. Sodium carbonate gives a blue color which is quite permanent.

Isolation of Glucose as Glucosazone.

The filtrate from the anthocyanidin chloride, mentioned above, was shaken with amyl alcohol until the dissolved pigment was removed. An excess of lead carbonate was added and the lead salts were filtered off and washed with water. The filtrate was freed from lead with hydrogen sulfide and after filtering off the lead sulfide, the solution was neutralized with sodium hydroxide and concentrated under reduced pressure to about 15 cc. This concentrated solution was mixed with 1.5 gm. of phenylhydrazine and 1.5 cc. of 50 per cent acetic acid dissolved in 4.5 cc. of water. After the solution had been heated for 15 minutes on the water bath the glucosazone began to crystallize in fine delicate needles. The crystals were filtered off after the solution had been heated for 1 hour and washed with water. The

crystals were dissolved in 70 cc. of hot 50 per cent alcohol, treated with norit, filtered, and allowed to crystallize. The solution deposited, on cooling, fine delicate yellow needles. They were filtered, washed with water, and dried in vacuum over sulfuric acid. The dry substance weighed 0.15 gm. Heated rapidly in a capillary tube the substance melted with decomposition at 204° (uncorrected). It is evident, therefore, that the sugar contained in the glucoside is dextrose.

Quantitative Determination of Anthocyanidin Chloride and Glucose.

The hydrolysis was carried out as described in the first paper.[1] From 0.7885 gm. of dry anthocyanin chloride we recovered 0.5236 gm. or 95.75 per cent of dried anthocyanidin chloride and 0.2498 gm. or 93.03 per cent of glucose. Calculated according to the equation, $C_{23}H_{25}O_{12}Cl+H_2O = C_{17}H_{15}O_7Cl+C_6H_{12}O_6$: anthocyanidin chloride 0.5468 gm., glucose 0.2685 gm.

The results mentioned above show that the anthocyanin chloride is a monoglucoside which yields 1 molecule each of anthocyanidin chloride and glucose when hydrolyzed by boiling hydrochloric acid.

Methoxyl Determination and Isolation of Delphinidin Iodide.

In the Zeisel determinations the dried anthocyanidin chloride was boiled with 9 cc. of hydriodic acid, sp. gr. 1.70, and 3 gm. of crystalline phenol.

Analyses. 0.2293, 0.3011, and 0.2227 gm. substance: 0.2124, 0.2707, and 0.2091 gm. AgI.

Calculated for 2 CH_3O. CH_3O 16.91 per cent.

Found. CH_3O 12.22, 11.87, and 12.40 per cent.

The values for methoxyl were lower than the calculated value for 2 CH_3O, but they were much higher than we had found in the anthocyanidin isolated from American grapes. The results indicate that the anthocyanidin under investigation consists largely of a dimethyl ether of delphinidin, but it is evident that it also contains some monomethyl ether of delphinidin.

Isolation of Delphinidin Iodide.

The mixture of hydriodic acid and phenol, containing the demethylated pigment, deposited, on cooling, prismatic crystals of

delphinidin iodide. The crystals were filtered, washed with ether, and dried in the air. The total amount obtained from the various determinations mentioned above weighed 0.4 gm. The crystals were of reddish brown color and showed a bronze luster. The substance was recrystallized as follows: It was dissolved in a little water acidified with hydriodic acid in which it formed a dark red solution. An equal volume of hydriodic acid, sp. gr. 1.70, was added. On standing for several hours at room temperature the substance crystallized in long prisms. The crystals were filtered, washed with absolute ether, and dried in a vacuum over sulfuric acid and potassium hydroxide.

Delphinidin iodide dissolves easily in water with a brownish red color, but a precipitate soon forms that dissolves on adding a few drops of acid, giving a deep red solution. This solution gives with ammonium hydroxide or sodium carbonate a greenish blue color. Sodium acetate gives a blue color. Sodium hydroxide produces a dirty green color that changes to yellowish brown and on acidifying the original color is not restored. Lead acetate gives a deep blue precipitate and copper sulfate gives a purple precipitate. The picrate separates slowly from a concentrated aqueous solution, on addition of picric acid, in bundles of thick reddish brown prisms. Delphinidin iodide dissolves in alcohol with purplish red color which on addition of ferric chloride turns deep blue.

The recrystallized delphinidin iodide was analyzed after it had been dried at 105° in a vacuum over phosphorus pentoxide. The loss in weight on drying was 7.40 and 7.37 per cent which corresponds to 2 molecules of water of crystallization. Calculated for 2 H_2O: H_2O 7.72 per cent.

Analyses. 0.1462 gm. substance: 0.0380 gm. H_2O and 0.2243 gm. CO_2. 0.1517 gm. substance: 0.0843 gm. AgI.

Calculated for $C_{15}H_{11}O_7I$ (429.9). C 41.87, H 2.55, I 29.52 per cent.
Found. C 41.84, H 2.90, I 30.03 per cent.

Anthocyanidin Picrate.

When an aqueous solution of anthocyanidin chloride is mixed with a saturated aqueous solution of picric acid the picrate is precipitated as dense irregular particles that show no definite crystalline structure. The picrate is very slightly soluble in water, but it is readily soluble in methyl alcohol and it crystallizes in

long needles when this solution is concentrated. About 0.5 gm. of the picrate was dissolved in 30 cc. of warm methyl alcohol and the solution was concentrated to 10 cc. on the water bath. The substance crystallized in very long fine needles from the warm solution. After the solution had cooled the crystals were filtered and washed with cold methyl alcohol and allowed to dry in the air. The dry substance was of dull brownish red color and it weighed 0.3 gm. It dissolved in methyl alcohol with deep purplish red color. It was only slightly soluble in ethyl alcohol, giving a beautiful purplish red color. It was nearly insoluble in water.

The substance was analyzed after it had been dried at 105° in a vacuum over phosphorus pentoxide. The loss in weight on drying was 3.78 and 3.98 per cent. Calculated for 1 H_2O: H_2O 3.11 per cent.

Analyses. 0.1244 gm. substance: 0.0377 gm. H_2O and 0.2255 gm. CO_2. 0.1133 gm. substance: N 8.00 cc. at 23° and 742 mm.

Calculated for $C_{17}H_{14}O_7 \cdot C_6H_2(NO_2)_3OH$ (559). C 49.37, H 3.04, N 7.51 per cent.

Found. C 49.44, H 3.39, N 7.94 per cent.

Absorption Spectra of Anthocyanin and Anthocyanidin Chlorides.

The absorption spectra given by these pigments are very similar to those reported in our earlier papers. The spectrum consists of one broad band extending from the yellow into the blue.

Anthocyanin Chloride.

Column.	In methyl alcohol. 1 molecule in 1,522 liters.	In ethyl alcohol. 1 molecule in 1,160 liters.
mm.		
2	575..566....515	585..569....525
3	580..571....505	
5	585..576....486	592..583....493
8		597..587....490
10		599..590....485

Anthocyanidin Chloride. 1 Molecule in 2,000 Liters.

Column.	In methyl alcohol.	In ethyl alcohol.
mm.		
3	556..549....497	562..557....503
5	561..552....487	567..561....484
7	564..554....478	570..565....471
10	565..558....461	572..567....—

SUMMARY.

The anthocyan occurring in Seibel grapes is a monoglucoside and it appears to be identical with oenin, the glucoside derived from *Vitis vinifera*. The pigment was isolated as the picrate which crystallized in red needles. The anthocyanin chloride crystallized in long prisms and corresponds to the formula $C_{23}H_{25}O_{12}Cl+3H_2O$.

The anthocyanidin chloride, $C_{17}H_{15}O_7Cl+1.5\ H_2O$, crystallized in prisms from the hot solution on boiling the glucoside with 20 per cent hydrochloric acid. The values obtained for methoxyl indicate that the anthocyanidin consists largely of a dimethyl ether of delphinidin, but evidently some monomethyl ether of delphinidin is also present. The substance is very similar to or identical with the oenidin obtained by Willstätter and Zollinger from *Vitis vinifera*.

The absorption spectra of the glucoside and of the sugar-free pigment consist of one broad band extending from the yellow into the blue.

Attention is drawn to the fact that in a cross between European and American varieties of grapes, such as the Seibel seedlings, the pigment peculiar to *Vitis vinifera* is inherited by the hybrid.

STUDIES OF ACIDOSIS.

XX. THE COLORIMETRIC DETERMINATION OF BLOOD pH AT BODY TEMPERATURE WITHOUT BUFFER STANDARDS.

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This paper has for its purpose, first, a study of the method of determining pH without buffer standards, and its application to blood pH determinations; second, a comparison of colorimetric and electrometric determinations of blood pH at body temperature; and third, an analysis of the variables entering into the correction factor used by Cullen (1) to convert colorimetric pH values at 20° into electrometric pH values at 38°.

Bjerrum (2) first introduced the bicolor principle for determining pH without standard buffer solutions, by employing two wedge-shaped glass vessels superimposed upon each other, one of which contained an indicator in acid solution, while the other contained the same indicator in alkaline solution. By looking through the superimposed wedges at different points he observed mixed colors equal to those of buffered solutions of certain definite pH values. Myers (3) has recently utilized this principle in devising a wedge colorimeter which is useful for the determination of the pH of small amounts of material with a high degree of accuracy.

Gillespie (4) showed that the Clark and Lubs series of indicators could be used in this way by combining pairs of test-tubes containing the "acid form" and the "alkaline form" of the indicators in definite ratios and that the pH corresponding to each ratio was given by the mass law equation in the logarithmic form

$$\mathrm{pH} = \mathrm{pK}' + \log \frac{\text{alkaline form}}{\text{acid form}} \qquad \text{(I)}$$

Michaelis (5) has used this principle for the determination of pH by his series of monocolor indicators, the nitrophenols and phenolphthalein.

Up to the present the bicolor principle has been employed only in pH determinations where standards at intervals of less than 0.20 pH were unnecessary. We have found that phenol red, the

indicator recommended by Cullen for the colorimetric determination of the reaction of blood, may be used for the preparation of standards by the bicolor method at 0.05 pH intervals, thereby permitting the estimation of colorimetric pH values to 0.02 pH. This corresponds to the accuracy possible with phosphate standards.

The advantages of the bicolor standards are stability, reproducibility, freedom from temperature effects, and simplicity of preparation.

Method.

Preparation of Standards.

The solutions required are phenolsulfonphthalein (phenol red) of known concentration, dilute alkali, *e.g.* 0.01 N NaOH, and dilute acid, *e.g.* 0.0001 N HCl. The only apparatus needed is a micro burette, a sufficient number of clear glass test-tubes of the same internal diameter, and a 3-row wooden comparator block of convenient size. The concentrations of indicator in the acid and alkaline solutions should be such that the total amount of dye in each pair of tubes is constant and provides a color depth convenient for accurate reading.

We have found the following concentrations of indicator to be satisfactory. A stock solution of phenol red, 0.1 per cent, made up as recommended by Clark (6), is kept on hand. When standard solutions are to be prepared, 15 cc. of the 0.1 per cent solution are diluted to 200 cc., yielding a 0.0075 per cent solution. In each pair of standard tubes the total amount of this indicator solution is 2.5 cc. The total volume of solution in each tube is 25 cc. Table I shows the amounts of 0.0075 per cent indicator solution to be added to the alkaline and acid tubes, respectively, to give the combined color effect corresponding to certain pH values.

The pH corresponding to each $\frac{BA}{HA}$ ratio has been determined by comparing tubes made up in the manner described above with standard phosphate solutions containing the same concentration of indicator. This comparison was made at 20° and 38°C. The results of these experiments are given in Table II and Fig. 1.

The curves are drawn from the equations

$$pH_{20°} = 7.78 + \log \frac{\text{alkaline form}}{\text{acid form}} \text{ and } pH_{38°} = 7.65 + \log \frac{\text{alkaline form}}{\text{acid form}}$$

where 7.78 and 7.65 are the values of pK′ of the dye at 20° and 38°, respectively, determined in the manner outlined later. The crosses, representing the experimental readings, show complete

TABLE I.

Table of pH Values at 20° and 38° at 0.05 Intervals, with Corresponding Amounts of 0.0075 Per Cent Phenol Red and 0.01 N NaOH or 0.0001 N HCl.

$pH_{20°}$	Alkali tube.		Acid tube.		$pH_{38°}$	Alkali tube.		Acid tube.	
	cc. dye	*cc. alkali*	*cc. dye*	*cc. acid*		*cc. dye*	*cc. alkali*	*cc. dye*	*cc. acid*
6.70	0.19	24.81	2.31	22.69	6.70	0.25	24.75	2.25	22.75
6.75	0.21	24.79	2.29	22.71	6.75	0.28	24.72	2.22	22.78
6.80	0.24	24.76	2.26	22.74	6.80	0.31	24.69	2.19	22.81
6.85	0.26	24.74	2.24	22.76	6.85	0.34	24.66	2.16	22.84
6.90	0.29	24.71	2.21	22.79	6.90	0.38	24.62	2.12	22.88
6.95	0.32	24.68	2.18	22.82	6.95	0.42	24.58	2.08	22.92
7.00	0.36	24.64	2.14	22.86	7.00	0.46	24.54	2.04	22.96
7.05	0.39	24.61	2.11	22.89	7.05	0.50	24.50	2.00	23.00
7.10	0.43	24.57	2.07	22.93	7.10	0.55	24.45	1.95	23.05
7.15	0.48	24.52	2.02	22.98	7.15	0.60	24.40	1.90	23.10
7.20	0.52	24.48	1.98	23.02	7.20	0.65	24.35	1.85	23.15
7.25	0.57	24.43	1.93	23.07	7.25	0.71	24.29	1.79	23.21
7.30	0.62	24.38	1.88	23.12	7.30	0.77	24.23	1.73	23.27
7.35	0.68	24.32	1.82	23.18	7.35	0.84	24.16	1.66	23.34
7.40	0.74	24.26	1.76	23.24	7.40	0.90	24.10	1.60	23.40
7.45	0.80	24.20	1.70	23.30	7.45	0.97	24.03	1.53	23.47
7.50	0.86	24.14	1.64	23.36	7.50	1.04	23.96	1.46	23.54
7.55	0.93	24.07	1.57	23.43	7.55	1.11	23.89	1.39	23.61
7.60	1.00	24.00	1.50	23.50	7.60	1.18	23.82	1.32	23.68
7.65	1.07	23.93	1.43	23.57	7.65	1.25	23.75	1.25	23.75
7.70	1.14	23.86	1.36	23.64	7.70	1.32	23.68	1.18	23.82
7.75	1.21	23.79	1.29	23.71	7.75	1.39	23.61	1.11	23.89
7.80	1.28	23.72	1.22	23.78	7.80	1.46	23.54	1.04	23.96
7.85	1.35	23.65	1.15	23.85	7.85	1.53	23.47	0.97	24.03
7.90	1.42	23.58	1.08	23.92	7.90	1.60	23.40	0.90	24.10
7.95	1.49	23.51	1.01	23.99	7.95	1.67	23.33	0.83	24.17
8.00	1.56	23.44	0.94	24.06	8.00	1.73	23.27	0.77	24.23

consistency with the above equations. This consistency, as stated above, has been previously demonstrated by Gillespie, but the possibility of using this method to estimate pH values closer than 0.2 intervals has not before been emphasized.

Determination of the pH of Plasma or Serum.

The discussion below covers the technique used with 0.2 cc. samples of plasma and 0.4 cc. samples of whole blood. The only changes necessary when larger samples are taken are the use of

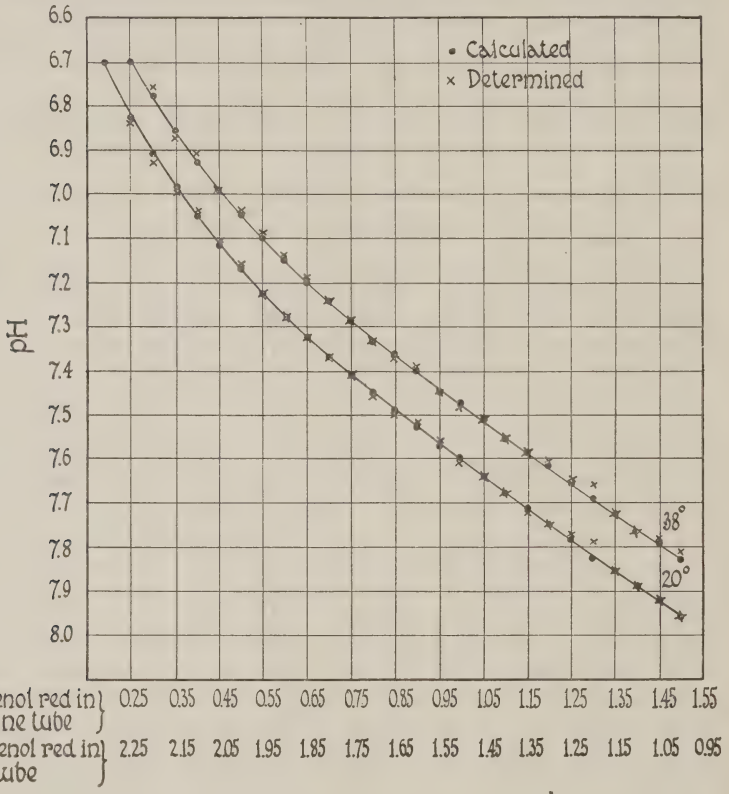

FIG. 1. The solid lines are drawn through the points satisfying the equations $pH_{20°} = 7.78 + \log \frac{\text{alkaline form}}{\text{acid form}}$ and $pH_{38°} = 7.65 + \log \frac{\text{alkaline form}}{\text{acid form}}$ respectively. The ×'s represent the readings actually obtained.

proportionally larger volumes of saline solution for dilution, and larger test-tubes.

4 cc. of adjusted saline indicator solution are pipetted under oil into a small test-tube (1.5 × 10 cm.). After addition of 0.2

cc. of serum or plasma, the mixture is gently stirred with a footed glass rod. A control tube is prepared in a similar manner, using saline solution without dye. The oil is replaced by paraffin, which

TABLE II.

Values of pK′ for Phenol Red Calculated at 20° and 38°.

BA	HA	$pH_{20°}$ observed.	$pK'_{20°}$ dye calculated.	$pK'_{20°}$ dye deviation from mean.	$pH_{38°}$ observed.	$pK'_{38°}$ calculated.	$pK'_{38°}$ deviation from mean.	$pH_{20°}$ calculated from Equation I.	$pH_{38°}$ calculated from Equation I.
cc. dye	*cc. dye*								
1.50	1.00	7.96	7.78	±0.00	7.81	7.63	−0.02	7.96	7.83
1.45	1.05	7.92	7.78	±0.00	7.78	7.64	−0.01	7.92	7.79
1.40	1.10	7.89	7.78	±0.00	7.76	7.65	±0.00	7.89	7.76
1.35	1.15	7.85	7.78	±0.00	7.72	7.65	±0.00	7.85	7.72
1.30	1.20	7.79	7.75	−0.03	7.66	7.62	−0.03	7.82	7.69
1.25	1.25	7.77	7.77	−0.01	7.64	7.64	−0.01	7.78	7.65
1.20	1.30	7.74	7.78	±0.00	7.60	7.64	−0.01	7.74	7.61
1.15	1.35	7.72	7.79	+0.01	7.58	7.65	±0.00	7.71	7.58
1.10	1.40	7.68	7.78	±0.00	7.55	7.65	±0.00	7.68	7.55
1.05	1.45	7.64	7.78	±0.00	7.51	7.65	±0.00	7.64	7.51
1.00	1.50	7.61	7.79	+0.01	7.49	7.67	+0.02	7.60	7.47
0.95	1.55	7.56	7.77	−0.01	7.44	7.65	±0.00	7.57	7.44
0.90	1.60	7.52	7.77	−0.01	7.39	7.64	−0.01	7.53	7.40
0.85	1.65	7.50	7.79	+0.01	7.37	7.66	+0.01	7.49	7.36
0.80	1.70	7.46	7.79	+0.01	7.33	7.66	+0.01	7.45	7.32
0.75	1.75	7.41	7.78	±0.00	7.28	7.65	±0.00	7.41	7.28
0.70	1.80	7.37	7.78	±0.00	7.24	7.65	±0.00	7.37	7.24
0.65	1.85	7.33	7.78	±0.00	7.20	7.65	±0.00	7.33	7.20
0.60	1.90	7.28	7.78	±0.00	7.14	7.64	−0.01	7.28	7.15
0.55	1.95	7.23	7.78	±0.00	7.09	7.64	−0.01	7.23	7.10
0.50	2.00	7.17	7.77	−0.01	7.04	7.64	−0.01	7.18	7.05
0.45	2.05	7.11	7.77	−0.01	6.99	7.65	±0.00	7.12	6.99
0.40	2.10	7.05	7.77	−0.01	6.91	7.63	−0.02	7.06	6.93
0.35	2.15	7.00	7.79	+0.01	6.87	7.66	+0.01	6.99	6.86
0.30	2.20	6.93	7.80	+0.02	6.81	7.68	+0.03	6.91	6.78
0.25	2.25	6.84	7.79	+0.01				6.83	6.70
Mean.....			7.78	−0.0004		7.65	−0.0024		

is allowed to solidify, and the tube of unknown pH is brought to a temperature 1° to 0.5° higher than required (usually to 39° or 38.5°), by immersion in a water bath. It is then placed in the comparator block, to be read when the temperature has fallen

to 38°. The block should have three rows of holes to admit tubes placed between the eye and the source of light in the manner indicated in Fig. 2. The known group, consisting of an alkaline tube, acid tube, and control tube is then compared with the unknown group, consisting of two water tubes and the unknown tube. By careful comparison, using standards at 0.05 pH intervals, one may interpolate as accurately as when but two tubes are used with buffer standards; *i.e.*, to 0.02 pH. 5 cc. portions of the bicolor standards prepared as above are placed in the tubes used for the comparison with unknowns. The above amounts of sample and saline are one-fifth of the amounts used by Cullen.

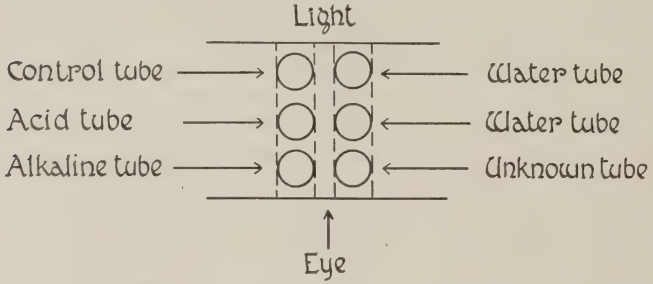

Fig. 2. Arrangement of tubes in comparator for reading colorimetric pH of blood by means of the bicolor standards.

Determination of the pH of Plasma in a Sample of Whole Blood.

This is essentially the technique proposed by Hawkins (7). Instead of 0.2 cc. of serum, 0.4 cc. of whole blood is taken as a sample and added to adjusted saline, the indicator content of which has been corrected as described in the next paragraph, for the increased volume of approximately 0.2 cc. due to the cells. The mixture is stirred and centrifuged, and the pH is determined as above. In this determination, it is necessary that the mineral oil be replaced by paraffin before centrifugation.

Saline Indicator Solution.

This solution, which contains per liter 0.154 M NaCl and 0.0000222 M phenol red, is prepared as follows: 0.9 gm. of NaCl is dissolved in freshly redistilled water in a 100 cc. flask. 10.50 cc. of 0.0075 per cent phenol red solution are added if the saline is to be

used in plasma determinations, 11.00 cc. if it is to be used for whole blood. The mixture is then diluted to the 100 cc. mark. A 0.154 M solution of NaCl without phenol red is prepared at the same time for control tubes.

The saline is adjusted to the approximate blood reaction as follows: At the beginning of each experiment, after addition of the phenol red, as indicated above, the solution is covered with paraffin oil. 0.01 N NaOH is admitted by capillary pipette, with stirring until approximately pH 7.4 is reached. Saline adjusted in this manner is hardly changed by standing overnight.

Control of Temperature.

Since the temperature has no effect on the colors of the alkaline and acid standard solutions, one needs only to control the temperature of the tube containing the serum. This may be simply done by immersing the tube in a beaker and gently heating until the desired temperature is reached. It is essential that a thermometer, held in place by a stopper so that it does not touch the sides of the tube, be used to determine the exact temperature of the solution. When the unknown solution is covered with solid paraffin, its temperature may be obtained by using the thermometer on either the control, or one of the water tubes, which has had exactly the same treatment as the unknown.

EXPERIMENTAL.

Effect of NaCl on Bicolor Standards.

Since in the determination of its pH, serum is diluted with 0.154 M NaCl (0.9 per cent) the effect of salt in this concentration on the bicolor standards was determined. This was done by making up two sets of bicolor standards, one in water and the other in 0.154 M NaCl. The concentrations of indicator, alkali, and acid were those used in the previous experiments. The tubes were matched against phosphate standards having the same amount of dye. The results of this experiment, given in Table III, indicate that in the presence of 0.154 M NaCl the bicolor standards are 0.01 to 0.02 pH more acid in appearance

than when no salt is present. A more complete study of the effect of salts in varying ionic strengths on the activity of the dye is in progress.

Determination of the pK′ of Phenol Red.

Table II indicates how this value, at 20° and 38°, was obtained. The pH values of bicolor standards made up in water were determined by comparison with phosphate standards. By substituting the value of the pH observed in Equation I, pK′ was calculated for each pair of bicolor tubes. The values 7.78 at 20° and 7.65 at 38° represent the mean of all such calculations of the pK′ of the dye at the two temperatures.

TABLE III.

Effect of 0.154 M *NaCl on the Bicolor Standards.*

Temperature.	pH of standard.		Salt "error."
	NaCl absent.	NaCl present.	
20°	7.38	7.36	0.02
	7.45	7.44	0.01
	7.53	7.52	0.01
	7.59	7.61	0.02
38°	7.14	7.14	0.00
	7.24	7.23	0.01
	7.33	7.30	0.03
	7.39	7.36	0.03
Average			0.016

As an incidental result of the previous experiment on the effect of NaCl, one may calculate the pK′ of phenol red in the absence of salt. Due to the effect of the phosphate on the activity of the salt of phenol red in the alkaline tubes, the color of the phosphate tube corresponded to that of a solution more acid by 0.02 than the real pH. Therefore, under conditions of zero salt the $pK'_{20°}$ of phenol red would be 7.76. Similarly, the $pK'_{38°}$ would be 7.63.

Comparison of Macro and Micro Determinations.

The desirability of using as little blood as possible for the determination of pH led Hawkins to the demonstration that

Cullen's method could be modified and successfully used on 0.25 cc. of blood. Several comparisons between the Cullen and Hawkins[1] methods have been made in this laboratory with satis-

TABLE IV.

Comparison of pH Determinations of Serum Made by Phosphate Standards and Bicolor Standards on 0.2 Cc. Samples.

No.	pH readings.	
	Phosphate standards.	Bicolor standards.
1	7.41	7.42
2	7.42	7.42
3	7.30	7.29
4	7.32	7.33

TABLE V.

Effect of Standing on Bicolor Standards.

pH on June 2, 1924.	pH on June 10, 1924.	Change in pH.
7.96	7.95	−0.01
7.92	7.92	±0.00
7.89	7.90	+0.01
7.85	7.88	+0.03
7.79	7.79	±0.00
7.77	7.77	±0.00
7.74	7.74	±0.00
7.72	7.72	±0.00
7.68	7.68	±0.00
7.64	7.64	±0.00
7.61	7.62	+0 01
7.56	7.56	±0.00
7.50	7.49	−0.01
7.46	7.47	+0.01
7.41	7.43	+0.02
7.17	7.17	±0.00
7.11	7.12	+0.01
6.93	6.94	+0.01

factory agreement between the two methods. To determine whether the bicolor standards could be used as successfully in the micro method as in the macro method, pH determinations of serum made with phosphate standards and those made with

[1] When pH determinations are made in the presence of cells, as in the Hawkins method, it is imperative that readings be made within half an hour after centrifugation, otherwise errors due to acid formation will occur.

bicolor standards in small test-tubes were compared. The results of a few such comparisons given in Table IV show satisfactory agreement between the two methods.

Stability of Bicolor Standards.

Although standard tubes of phosphate and phenol red are not reliable for use in blood pH determinations after 3 days, we have found that the bicolor standards remain practically unchanged after 8 days (Table V). The maximum length of time for which the standards may be regarded as reliable is as yet undetermined.

Comparison of Electrometric and Colorimetric pH Values at 38°.

A series of experiments was made in which the pH values of plasma from the blood of various species, at different CO_2 tensions, were determined both electrometrically and colorimetrically at 38°. These results are given in Table VI. The species studied were man, horse, dog, and rat. In all, twenty-three such experiments have been performed with the result that the average difference between the determinations made in the two different ways is -0.003 with a maximum difference of ± 0.04. The differences encountered are not outside the combined errors of the two methods and it may therefore be concluded that, within the limits of experimental accuracy, the colorimetric pH determinations by Cullen's method *when read at 38°* are identical with electrometric pH determinations. That this identity is real and not accidental is shown by the fact that the plasma of two species, the dog and rat, which gave at room temperature such widely different Cullen corrections as 0.35 and 0.12, respectively, show no differences between their colorimetric and electrometric values at the same temperature.

Standard Phosphates at 38°.

In order to read colorimetric pH values at 38° it was necessary to have the standard pH solutions at the same temperature as the unknown, otherwise the question of the effect of temperature on the dye would be involved. The pH of standard phosphate solutions made up as described by Cullen was therefore determined electrometrically at 38°, using as the standard of reference

TABLE VI.

Comparison of Electrometric and Colorimetric pH Values.

Date.	Species.	No.	Electrometric 38°.	Colorimetric 38°.	$pH_e - pH_c$	Colorimetric method.
1924						
Apr. 9	Man.	1	7.34	7.33	+0.01	Cullen.
		2	7.53	7.53	±0.00	"
" 29	Horse.	1	7.31	7.33	−0.02	"
		2	7.46	7.47	−0.01	"
		3	7.32	7.34	−0.02	"
		4	7.46	7.47	−0.01	"
" 24	Dog.	1	7.25	7.27	−0.02	"
		2	7.40	7.41	−0.01	"
		3	7.26	7.27	−0.01	"
		4	7.40	7.40	±0.00	"
" 11	Rat.	1	7.01	6.99	+0.02	Hawkins.
		2	7.20	7.16	+0.04	"
		3	7.35	7.35	±0.00	"
" 10	Horse.	1	7.22	7.26	−0.04	Cullen.
		2	7.50	7.51	−0.01	"
May 28	"	1	7.52	7.52	±0.00	"
		2	7.50	7.49	+0.01	"
		3	7.35	7.36	−0.01	"
		4	7.33	7.34	−0.01	"
June 3	"	1	7.39	7.40	−0.01	Bicolor.
		2	7.39	7.41	−0.02	"
		3	7.21	7.23	−0.02	"
		4	7.22	7.25	−0.03	"
			Electrometric 20°.	Colorimetric 20°.		
Jan. 9	Horse.	1	7.27	7.26	+0.01	Cullen.
		2	7.46	7.46	±0.00	"
		3	7.72	7.69	+0.03	"
Apr. 8	Man.	1	7.15	7.14	+0.01	"
		2	7.37	7.36	+0.01	"
		3	7.56	7.57	−0.01	"
		4	7.62	7.60	+0.02	"
Average deviation					−0.003	
Maximum "					±0.04	

TABLE VII.

Electrometric pH Values at 20° and 38° of M/15 Phosphate Mixtures. Average Results of Two Experiments.

M/15 Na_2HPO_4	M/15 KH_2PO_4	pH determined at 20°.	pH determined at 38°.	pH 20°–38°	Deviation from mean difference.
cc.	*cc.*				
49.6	50.4	6.809	6.781	−0.028	+0.001
52.5	47.5	6.862	6.829	−0.033	−0.004
55.4	44.6	6.909	6.885	−0.024	+0.005
58.2	41.8	6.958	6.924	−0.034	−0.005
61.1	38.9	7.005	6.979	−0.026	+0.003
63.9	36.1	7.057	7.028	−0.029	±0.000
66.6	33.4	7.103	7.076	−0.027	+0.002
69.2	30.8	7.154	7.128	−0.026	+0.003
72.0	28.0	7.212	7.181	−0.031	−0.002
74.4	25.6	7.261	7.230	−0.031	−0.002
76.8	23.2	7.313	7.288	−0.025	+0.004
78.9	21.1	7.364	7.338	−0.026	+0.003
80.8	19.2	7.412	7.384	−0.028	+0.001
82.5	17.5	7.462	7.439	−0.023	+0.006
84.1	15.9	7.504	7.481	−0.023	+0.006
85.7	14.3	7.561	7.530	−0.031	−0.002
87.0	13.0	7.610	7.576	−0.034	−0.005
88.2	11.8	7.655	7.626	−0.029	±0.000
89.4	10.6	7.705	7.672	−0.033	−0.004
90.5	9.5	7.754	7.726	−0.028	+0.001
91.5	8.5	7.806	7.776	−0.030	−0.001
92.3	7.7	7.848	7.825	−0.023	+0.006
93.2	6.8	7.909	7.877	−0.032	−0.003
93.8	6.2	7.948	7.919	−0.029	±0.000
94.7	5.3	8.018	7.977	−0.041	−0.012
Mean difference				−0.029	
Average deviation from mean difference					+0.00004

TABLE VIII.

Error Involved in Failure to Adjust Saline.

Electrometric $pH_{20°}$.	Colorimetric $pH_{20°}$. Dilution = 21 times.	
	Saline adjusted.	Saline unadjusted.
7.27	7.26	7.24
7.46	7.46	7.45
7.72	7.69	7.66

the ϵ of the saturated calomel cell as determined with 0.1 N HCl and assuming as its pH 1.08, at 38°. Two complete series at 0.05 pH intervals were run. The results of these determinations are given in Table VII, which gives the ratio of Na_2HPO_4 and KH_2PO_4 corresponding to 0.05 pH intervals from pH 6.8 to 8.0. A comparison of these values with those obtained at 20° shows the latter to be 0.03 more alkaline at any given $\frac{Na_2HPO_4}{KH_2PO_4}$ ratio. Similar results have been obtained by Cullen.

Adjustment of Reaction of Saline Solution Used for Diluting Blood.

Cullen has emphasized the necessity of adjusting to 7.4 the pH of saline solution used in diluting the serum. The results of experiments given in Table VIII demonstrate the necessity of

TABLE IX.

Comparison of Mineral Oil and Paraffin in Preventing Loss of CO_2.

20°	Sample.	Date June 3, 1924.	Date June 4, 1924.	pH change overnight.
Paraffin	1 *a*	7.63	7.63	±0.00
Oil	*b*	7.62	7.66	+0.04
Paraffin	2 *a*	7.62	7.67	+0.05
Oil	*b*	7.61	7.64	+0.03
Paraffin	3 *a*	7.41	7.41	±0.00
Oil	*b*	7.41	7.48	+0.07
Paraffin	4 *a*	7.43	7.44	+0.01
Oil	*b*	7.44	7.50	+0.06

such adjustment. They show that when the pH of the serum is near that of the saline, the colorimetric pH at 21-fold dilution is within 0.01 to 0.03 of the electrometric pH at 20°, whereas the pH of the solution of serum in unadjusted saline is 0.02 to 0.06 less at the same dilution. At higher dilutions the discrepancy becomes greater.

Use of Paraffin to Cover Solutions.

Cullen originally recommended the use of oil to cover the solutions whose pH was to be determined. Hawkins prepared tubes

of such dimensions that they were entirely filled up to the stopper with solution. We have found that it is an advantage to replace the oil with paraffin after the serum or blood has been mixed with saline solution. Loss of CO_2 by passage from the solution into the oil introduces an error which, although not significant at room temperature, seems to be of measurable magnitude at 38°. Table IX shows the effect of allowing the tubes to stand overnight when covered with paraffin and with oil. As might be expected, the greater increase in alkalinity with standing is in those tubes with a higher initial CO_2 tension.

DISCUSSION.

Factors Involved in the Cullen Colorimetric Correction.

The Salt Error.—Among the factors composing the colorimetric correction, the salt error of the dye might have been conceived to play a rôle. This is negligible in the determinations as carried out by Cullen's method. It will be shown in a later paper that the so called salt error of phenol red is really a change in the activity of the ions which is a function of the ionic strength of the solutions (Lewis and Randall, 8). The ionic strength of the saline solution is 0.154 μ and of the phosphate standards, 0.134 μ at 6.8 and 0.190 μ at 7.8. Thus the ionic strengths of the standard and unknown solutions are approximately the same and any effect produced on the activity of the dissociated dye will be the same in both solutions.

The Protein Error.—Although the influence of proteins on the color of phenol red is great in undiluted serum or plasma, at the 21-fold dilution which Cullen chose as the optimum dilution for reading colorimetric serum pH, this error is negligible.

The Dilution Error.—It is, of course, conceivable that diluting a solution with a non-buffered NaCl solution will lead to a pH which is different from that of the original undiluted solution; *i.e.*, there may be an error in the resulting pH introduced by dilution. This possibility we have tested in the following manner.

Solutions, 30 mM with respect to $NaHCO_3$ and 100 mM with respect to NaCl, were equilibrated with CO_2 at 38°. After dilution ranging from 0- to 21-fold with adjusted, isotonic NaCl solution, the pH of each solution was determined colorimetrically at 20° and 38°. The results of this experiment are given in Table X. The pH was not significantly changed by dilution in any case.

One may conclude, therefore, that 21-fold dilution, *per se*, with an isohydrionic, isotonic NaCl solution has no measurable effect upon the pH of the solution.

Effect of Temperature.

Since the system contains not only $BHCO_3$ and H_2CO_3 but also proteins and their salts, variations of the temperature effect from that of a simple $BHCO_3$-H_2CO_3 solution are to be expected. In plasma we have from this standpoint the following equilibria

$$H_2CO_3 = \overset{+}{H} + \overset{-}{HCO_3} + 2{,}800 \text{ cal. (Landolt-Börnstein, 9)}$$

$$HP = \overset{+}{H} + \overset{-}{P} + 10{,}000 \text{ cal. approximately (Meyerhof, 10)}$$

TABLE X.

Effect of Dilution with Isotonic NaCl on the pH of Bicarbonate Solutions at 20° and 38° C.

Solution.	Dilution.	Colorimetric pH.		Difference.
		20°	38°	
A	0	7.65	7.60	0.05
	5	7.64	7.60	0.04
	10	7.64	7.60	0.04
	15	7.65	7.60	0.05
	21	7.64	7.59	0.05
B	0	7.68	7.63	0.05
	5	7.68	7.62	0.06
	10	7.66	7.61	0.05
	15	7.66	7.61	0.05
	21	7.65	7.60	0.05

According to the van't Hoff isochore the change in $pK'_{H_2CO_3}$ from 38° to 20° should be +0.12 and in pK'_{HP}, +0.43. The difference in the pH of plasma at 38° and at 20° would therefore lie somewhere between 0.12 and 0.43, with the occurrence of variations in this difference depending on the character and concentration of the serum proteins. The temperature effects observed by Cullen are within these limits. The variations in the

effects with species and pathological condition are comprehensible, as well as the desirability of avoiding such variations by performing the determinations at body temperature.

SUMMARY.

Using pairs of tubes to give varying ratios of the alkaline and acid forms of the indicator, it has been demonstrated that phenol red in bufferless solutions may be successfully used in place of phosphate standards, for the estimation of the reaction of blood or serum to within ± 0.02 pH.

It has been found that colorimetric and electrometric determinations of the pH made on the same sample of blood agree to within 0.02 pH when both determinations are made at the same temperature. The correction used by Cullen for colorimetric determinations performed at room temperature is unnecessary when the readings are made with the diluted plasma at body temperature.

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THE EXTRACTION AND SOME PROPERTIES OF AN OVARIAN HORMONE.* †

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HISTORICAL.

Perhaps the first good experimental evidence that the ovary produces an internal secretion which governs the phenomena of estrus may be attributed to Knauer (1900). Although the atrophy of the uterus following removal of the ovaries had been established much earlier, the proof that the relationship is not nervous but is by way of the blood stream lies in the autotransplantation experiments of Knauer. By successfully grafting the ovaries in abnormal positions he showed that the uterus and secondary sex characteristics retained their normal characters. These experiments have been verified and extended by other investigators (Marshall and Jolly, 1905).

In view of the numerous extensive reviews (Herrmann, 1915; Novak, 1922; Frank, 1922; Marshall, 1923) of the subject of internal secretions of the ovary a complete discussion of the literature is superfluous. A few papers which are closely related to our work must be considered in some detail.

The utilization of organic solvents for extraction of the hormone from ovarian tissue (which we have found to be very important) was introduced by Iscovesco (1912, 1914). The preparation described by him is based upon a laborious separation of various fractions of lipoids by their differ-

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† The authors take this opportunity of acknowledging the assistance of Mr. W. B. Kountz and Mr. H. V. Gibson of Washington University during the earlier stages of the work described in this paper.

ential solubilities in organic solvents. Although we have no doubt that Iscovesco obtained active preparations in some of his fractions we would criticize his experiments for two reasons: (1) he used normal adult rabbits as test animals; the ordinary phenomena of "heat" due to ovarian influence include hyperplasia of the uterus and thereby introduce uncertainty in the interpretation of his positive results; and (2) he believed administration of the extracts by mouth to be effective; this we believe to be erroneous, it being our experience that preparations which are active if given subcutaneously are inactive on oral administration.

Fellner (1912, 1913), who published a preliminary paper shortly after Iscovesco's first paper appeared, likewise used organic solvents. Although Fellner's work is far from conclusive, it must be regarded, because of his improved method of testing, as the first to demonstrate that an active extract of the hormone may be obtained by alcoholic extraction of ovaries or placenta. The alcohol was evaporated, the residue suspended in saline and injected. This preparation produced hyperplasia in the uterus and vagina of spayed rabbits. It seems that Fellner did not attempt to purify this crude extract. Injections of it frequently produced severe disturbances in rabbits and in many cases parenchymatous nephritis. It is unfortunate that Fellner did not push his promising observations to a logical conclusion.

Fellner's contributions may be summed up as follows: (1) extraction by alcohol of a substance from ovaries and placenta which produced hyperplasia in ovariectomized rabbits; and (2) evidence that this substance is soluble in alcohol, acetone, and ether and that it is thermostable.

The criticisms which may legitimately be offered are: (1) the crude extract is not suitable for injection into patients and no attempt to purify the hormone has been published in the succeeding decade; and (2) emphasis on the importance of using ovaries containing corpora lutea which according to our work is quite erroneous.

In chronological order Okintschitz (1914) may be considered next. He made extracts from whole ovaries ("ovariin"), liquor folliculi ("proprovar"), and corpora lutea ("luteovar") by grinding the tissue with saline and glycerol. After 3 days the suspension was filtered and the activity of the filtrate tested. By subcutaneous injection he showed that extracts of liquor folliculi or whole ovaries retarded the rate of atrophy of the uterus of ovariectomized rabbits whereas the extract of corpora lutea had no effect. Strangely enough, although he used methods of extraction and animal tests which we consider inadequate, Okintschitz drew the correct conclusions. Instead of merely retarding atrophy in spayed animals active extracts should produce a marked degree of hyperplasia. Okintschitz's paper does not appear convincing and is mentioned mainly because it appears that he detected a difference between extracts of corpus luteum and the liquor folliculi or whole ovaries.

Probably the most elaborate investigations in the field of ovarian hormones have been conducted by Herrmann and Fränkel. Herrmann (1915), using organic solvents combined with fractional distillation *in vacuo*, obtained a product which he claimed to be the hormone of the

placenta in pure form. At the same time he showed that injections of this "pure" hormone produced the *same effect as purified extracts from the corpora lutea.* Subsequently Fränkel and Fonda (1923) isolated a substance from *corpora lutea* which had the same physical, chemical, and physiological properties as the substance obtained by Herrmann from the placenta. Although we have not used fractional distillation *in vacuo,* the other steps of the preparation described by Herrmann are very similar to the procedure adopted by us and in view of this similarity of method and our definitely positive results it is quite probable that Herrmann's extracts from placenta contained active material.

Fränkel and Herrmann have taken out a patent in the United States (U. S. 1,314,321), Germany (D. R. P. 309,482 and 309,606), and possibly other countries. Although we have inquired we have not found any pharmaceutical house in the United States operating under the Fränkel-Herrmann patent. Whether this means that the price asked is too high or that the investigations of the manufacturers have not confirmed the patent specifications we have no means of knowing. A more complete discussion of the patent and scientific papers is given later in this paper in the comparison of our work with that of Fränkel and Herrmann.

Frank (1917) and his collaborators, believing in the efficacy of lipoid solvents for extraction of the active principle of the ovary, have published a number of papers. While they (Frank and Rosenbloom, 1915) extracted a growth-producing substance from the placenta, they likewise claim to have found one in the corpora lutea.

Although all of the authors referred to in the preceding paragraphs who used lipoid solvents may have obtained active extracts from whole ovaries or placenta, their finding of the hyperplastic substance in the corpora lutea casts doubt upon their observations. While it is not our province to explain this confused situation, we may point out that in some cases it might be attributed to the use of normal animals in testing for activity. Certainly none of the authors with the possible exception of Herrmann has shown that this extract can restore to ovariectomized animals the full growth of the genital tract and the sexual manifestations characteristic of estrus.

INTRODUCTION.

Due to the confusion and uncertainty of the literature concerning ovarian hormones and influenced especially by the skeptical opinions expressed in recent reviews of the subject we paid but little attention to the papers of the various workers who have claimed to have demonstrated active preparations until after our own first definitely positive results were obtained.

Although, we may be accused of quoting Scripture for our purposes, a few excerpts from recent papers may be used to show the feeling in many quarters regarding the status of ovarian hormones.

From the standpoint of the clinician we quote the following excerpt from Frank's review:[1] "This leads me to state that today we have no better ovarian extract on the market than we had in 1910 when I first discussed this subject. The commercial preparations are 'degreased' or 'defatted' and therefore deprived of such minute doses of the active principle as they may have originally contained. Pharmacologically they are inert."

Again we read in Novak's paper:[2] "In spite of the confession of unsatisfactory results embodied in this paper, I am frank to say that I employ ovarian therapy for certain indications because I believe it to be based on rational principles, and because it is reasonable to hope that the biological chemist will sooner or later succeed in giving us ovarian extracts which will really approximate in their effects the action of the ovarian secretion intra vitam. This, after all, is the real crux of the situation as it now exists." And in another place:[3] "It cannot be said that there has been any noteworthy advance in ovarian therapy in the quarter century and more which has elapsed since its introduction. This is especially true as applied to the preparation of the commercial extracts upon which the profession as a whole is dependent."

We have expressions of skepticism from the experimental biologist as well as the clinican. Corner (1923) in his review[4] of "Oestrus, ovulation and menstruation" says: "The actual causal relationships, endocrinal or otherwise, between the follicle and corpus luteum on one hand and the uterus on the other, are as yet largely unexplored." And again:[5] "We have, no doubt, a long time to wait for chemical identification of the reproductive hormones and their exact sources; . . . . "

It is only since the preliminary stages of our work that we are able to pick out the papers mentioned in the historical section from a large number of others as those which come near to the true state of affairs. As in all other fields of endocrinology it was impossible at first to separate the chaff from the grain and with so many conflicting papers a clear view of the ovarian hormone field was impossible. We hope that we may be pardoned if we have been unduly severe in our criticisms.

[1] Frank (1922), p. 184.
[2] Novak (1922), p. 617.
[3] Novak (1922), p. 619.
[4] Corner (1923), p. 471.
[5] Corner (1923), p. 478.

EXPERIMENTAL.

Our first and most fundamental observation was that the injection of *fresh liquor folliculi* of pig ovaries into ovariectomized rats and mice caused *typical* estrus, the reaction being equal in degree to normal estrus from ovarian influences. Following this observation, we undertook to extract and separate the active principle and to determine its chemical and physical character. We have also studied the distribution of the hormone in various tissues.

Influenced by the successful extraction of insulin with alcohol by Banting, Best, Collip, and Macleod (1922), our first preparations were made by merely mixing liquor folliculi with alcohol, filtering, and driving off the alcohol from the filtrate with an air current. The residual aqueous suspension injected subcutaneously produced estrus in an ovariectomized rat. In attempting to remove the lipoids before injecting the aqueous residue, extraction with ether was resorted to. To our surprise the aqueous solution was no longer active, but the ethereal solution was. Following up this observation, we worked up the method of extraction which with only slight modifications has been in use for over a year. Consulting again the investigations which had been based on extraction with organic solvents, it was seen that our method of preparation was quite similar to the one described by Herrmann. There are, however, three important points on which we differ from Herrmann: namely, (1) the corpora lutea as a source of the hormone; (2) cholesterol reactions of the hormone; and (3) method of testing the physiological activity.

Herrmann claims to have obtained an extract of corpus luteum which induces hyperplasia of the uterus and vagina. Although we have used the same method that yields active extracts from liquor folliculi, whole ovaries, and placenta our results on more than twenty preparations from corpora lutea of both estrus and pregnancy have been uniformly negative. There appears to be a flat contradiction between the results of Herrmann and Fränkel and our own. Supposing that it might conceivably be due to a difference in the species of animals used we have tested some of our preparations on sexually immature rabbits and obtained no signs of activity.[6]

[6] Because of the stress which some authors place on the corpus luteum as a gland of internal secretion, Mr. C. G. Johnston and Mr. V. L. Gould will present the results of their work in a separate publication. With their

TABLE I.

Comparison of Activity of Hormone Preparations before and after Removal of Cholesterol.

No.	Source.	Before removal of cholesterol.			After removal of cholesterol.		
		Quantity injected.	Result of test.	Cholesterol content.	Quantity injected.	Result of test.	Cholesterol content.*
		mg.		*mg.*	*mg.*		*mg.*
97	Liquor folliculi.	1.71	+	0.83	0.60	++	0.00
		0.17	−	0.08	0.10	−	0.00
99	" "	1.00	+	0.16	0.62	+	0.00
		0.10	−	0.02	0.06	−	0.00
150 Y	" "	1.17	+	0.25	0.92	+	0.00
		1.02	−	0.21	0.80	−	0.00
150 Z	" "	0.43	+	0.25	0.18	+	0.00
		0.38	−	0.21	0.16	−	0.00
170 X†	" "	15.00	+	1.82	13.18	+	0.00
170 Y	" "	3.20	+	0.59	2.61	+	0.00
170 Z	" "	2.20	+	0.21	1.99	++	0.00
1840 A	" "	0.38	±	0.06	0.13	+	0.00‡

* The figures in this column are based upon the assumption of the complete removal of cholesterol by the digitonin process. Although this is not quite true, the colorimetric test showed that less than 0.01 mg. per rat unit remained.

† No further dilutions were made of this due to lack of material.

‡ The reduction in solids is greater than can be accounted for by the removal of cholesterol due to additional steps in purification. Calculation from our data shows that *less than* 0.0008 mg. of cholesterol or substances giving the cholesterol color reaction could be present per rat unit.

permission, we may say that they have extracted corpora lutea of various degrees of development in pregnant and non-pregnant animals by the method described in this paper. Injection of the extracts has always given negative results.

In most of their publications, Herrmann and Fränkel refer to the marked cholesterol reactions of their "pure hormone." The most obvious method of testing this point is to compare the physiological activity and color reactions of a preparation before and after removal of the cholesterol. In Table I, we present the results of this comparison. The partially purified extracts were analyzed by the colorimetric method of Bloor, Pelkan, and Allen (1922) and an excess of digitonin was added. The filtrate was evaporated and the residue extracted with ether. The ether was evaporated and this residue dissolved in chloroform was tested by the Salkowski and Liebermann-Burchard color reactions. Over 25 rat units (No. 1840 A) in 3 cc. of chloroform gave less color than 0.02 mg. of cholesterol; in fact, the presence of a positive color reaction was doubtful, yet the activity of the preparation was not appreciably altered. We feel that these experiments prove that the pure hormone of the liquor folliculi does not give cholesterol color reactions and that if this hormone is identical with the active substance of the placenta Fränkel and Herrmann must have been dealing with an impure product. A similar view has been expressed by Frank (1917) in his statement that cholesterol may be removed without destruction of the activity.

Again the quantities injected by Herrmann cast some doubt upon the purity of his product. The smallest dose of which he speaks is 60 mg., injected in two portions. In some of his rabbits weighing about 1 kilo 240 mg. were injected over a period of 10 days. These amounts of a pure hormone for the production of a physiological response are extraordinarily large. (Compare Dudley (1923), Abel, Rouiller, and Geiling (1923), posterior lobe; Shonle and Waldo (1924), Doisy and Weber (1924), insulin.) We have injected doses of 2, 4, and 8 mg. of one of our better preparations into young rabbits. It is obvious from Fig. 1 that 4 mg. has some effect and that 8 mg. has produced a pronounced enlargement.

Little need be said here concerning our method of testing the activity of extracts since this has been dealt with in detail in another paper.[7] From our experience, external examination of the vulva of the rabbit and rat for swelling and hyperemia is not satisfactory. We have used the induction of the manifestations of estrus in ovariectomized rats as the test of activity of our extracts. Since the growth phase of the estrus cycle in this animal is complete in 2 or 3 days it has been possible with three injections to get a maximal reaction in the genital tract instead of the partial reaction in the rabbit which has generally served as a criterion of activity for earlier investigators. Using the smear method of Stockard and Papanicolaou (1917), the course of the experiment can be followed in the living animal which can be used repeatedly.

[7] Allen, E., and Doisy, E. A., *Am. J. Anat.*, 1924, (in press).

The clear-cut nature of this test lessens the confusion of uncertain results. This improvement in testing has enabled us to make more than 600 separate tests of extracts in a little over 1 year with very little interference to our other work.

FIG. 1. × 0.40. Effect of injection of one of our better preparations into 8 week old rabbits. Animals were litter sisters. No. 807 received 2 mg.; No. 805 not injected and killed at the same time as No. 807 for control. No. 806 received 1 mg. daily for 4 days and No. 809 1 mg. daily for 8 days. No. 808, uninjected control, killed on same day as No. 809. No. 809 did not photograph well because of the pronounced hyperemia which decreased the contrast between the tissue and background.

Provisionally we wish to define a *rat unit* as the quantity of material necessary to induce estrus as judged by the smear method in an ovariectomized sexually mature rat weighing 140 ± 20 gm. For physiological reasons, we generally make three injections at intervals of 4 hours. The rat unit then is the minimum total amount so injected which produces full estrus growth in the geni-

tal tract. Although we are convinced that this method of testing is roughly quantitative, we have not yet made a careful study of its limitations. In our tables where we have given the yield in rat units, a generous latitude in either direction should be allowed. Realizing the possible inaccuracies in our assays, no attempt will be made to base conclusions on small quantitative differences.

Preparation of the Hormone from Liquor Folliculi.

In a preliminary paper (Allen and Doisy, 1923), we described a method of obtaining the hormone from liquor folliculi. Since that time we have introduced a few minor modifications which render the preparation less time-consuming.

Routine Method.

Fresh liquor folliculi obtained from hog ovaries by aspiration is mixed with 2 volumes of 95 per cent alcohol. After coagulation the proteins are filtered off and extracted with 95 per cent alcohol for about 6 hours in a Soxhlet tube. The filtrate and extract are combined and concentrated to dryness by either vacuum distillation or a current of warm air. We prefer the latter method and for this purpose use a ventilating fan which drives a current of warm air over the liquid exposed in shallow pans. The dry residue is emulsified with a few cubic centimeters of water and transferred to a flask. The pan is washed with alcohol and the emulsification with water repeated if necessary. Enough 95 per cent alcohol is added to make the total volume in the flask equivalent to about 15 cc. per 100 cc. of liquor folliculi extracted. The alcohol is heated to boiling, 2 volumes of acetone are added with shaking, and the mixture is cooled. The liquid is filtered off and the residue boiled again with the same volume of 95 per cent alcohol. The precipitation with acetone and filtration are repeated. Three or four such leachings are sufficient to remove all of the hormone from the solids which consist partly of phospholipins and partly of inorganic salts. The combined filtrates are distilled to dryness (Stage A) and the residue leached five to seven times with small volumes (20 cc. per liter of liquor) of boiling 95 per cent alcohol with subsequent cooling in a salt-

ice mixture before filtration. This step removes the hormone and leaves a considerable amount of fatty material, probably triglycerides, in the residue. The alcoholic solution is distilled to dryness and the residue taken up in anhydrous ether. There is usually some brown oily material which is insoluble in the ether and upon standing a white precipitate frequently forms. The ethereal solution is centrifuged and the precipitate rejected. This solution is our stock supply. It is evaporated and the residue dissolved in Mazola (purified corn oil) for injections. The various fractions which have been discarded have been tested and found to possess very little or no activity, but some of them are quite toxic.

Instead of liquor folliculi whole ovaries or placenta may be used in the preparation, but due to the greater quantity of material extracted by alcohol from these tissues greater quantities of solvents must be used at the different stages of extraction and purification.

The method described is our routine procedure with which several modifications have been compared. At stage A an advantageous purification may be effected by moistening the residue with warm alcohol and then boiling with acetone, cooling, and filtering. This extraction should be repeated three or four times. Quite a large proportion of the solids is eliminated with no loss of activity. The subsequent treatment of the acetone solution is the same as the other steps of the routine method. Reference to Table II shows that the preparations Nos. 64 and 184 appear to have given more rat units per kilo of liquor folliculi than extracts made by the routine method. Between 0.30 and 0.40 mg. of solids of each of these preparations was a rat unit. These and other data which are not presented here seem to indicate that the activity of the extracts may be increased by purification, but the evidence on this point is not conclusive.

Due to the nature of the compounds extracted by the solvents used we have attempted to use hydrolysis by acids and bases to shorten the work of preparation. Some of our products have been freed of a large proportion of the impurities, but in some instances the hormone has been destroyed. However, it appears to be fairly stable toward mild hydrolysis and we are continuing our study of this procedure as an aid to purification.

TABLE II.

Yield, Solid Content, and Activity of Hormone Preparations Made by Different Processes.

No.	Source.	Treatment.	Quantity injected.		Result of animal test.	Liquor folliculi yield in rat units per kg.
			Equivalent of liquor folliculi.	Solids.		
			cc.	*mg.*		
140 X	Liquor folliculi.	Routine.	2.1	5.00	+	480
			1.8	4.26	−	555
150 X	" "	"	2.3	4.37	+	430
			2.0	2.18	−	500
170 X	" "	"	4.5 *	15.0	++	220
175 A	" "	"	2.7	19.5	+	370
			1.3	9.4	−	770
174 A	" "	"	2.0	8.2	+	500
161	" "	Alcohol-ether precipitation of proteins and then routine.	2.5	1.40	+	400
			2.0	1.16	−	500
176	" "	Precipitation of proteins with boiling alcohol and then routine.	1.5	4.70	+	670
			0.75	2.4	±	1,340
64	" "	Routine with acetone at Stage A.	0.66	0.31	+	1,500
			0.33	0.15	−	3,000
184	" "	Same as No. 64.	0.91	0.75	+	1,100
			0.46	0.38	±	2,180
99	" "	Hydrolysis, H_2SO_4.	3.0	1.00	+	333
			0.3	0.10	−	3,333
1493	" "	" "	3.0	2.70	+	333
			1.0	0.90	−	1,000

TABLE II—*Concluded.*

No.	Source.	Treatment.	Quantity injected.		Result of animal test.	Liquor folliculi yield in rat units per kg.
			Equivalent of liquor folliculi.	Solids.		
			cc.	*mg.*		
140 Y	Liquor folliculi.	Hydrolysis, H_2SO_4.	3.2	4.00	+	310
			2.1	2.66	−	480
150 Y	" "	" "	2.3	1.17	+	430
			2.0	1.02	−	500
170 Y	" "	" 0.1 N HCl.	4.5 *	3.20	+	220
97	" "	" NaOH.	9.0	1.71	+	110
			0.9	0.17	−	1,110
110	" "	" "	2.00	1.88	+	500
			0.67	0.63	±	1,500
1492	" "	" Na_2CO_3.	3.0	2.70	+	333
			1.0	0.90	±	1,000
140 Z	" "	" NaOH.	3.2	5.75	+	310
			2.1	3.92	−	480
150 Z	" "	" "	2.3	0.43	+	430
			2.0	0.38	−	500
170 Z	" "	" "	4.5 *	2.20	++	220

* No material left for further dilution.

In the column headed "Result of animal test," + signified full estrus; ++, an abnormally prolonged estrus; ±, growth but not quite the attainment of full estrus; and −, no growth detectable by the smear method.

We have given the minimum quantity injected which returned a + and the maximum which gave a − result. In some cases (for example, Nos. 97 and 99) the assays were incomplete with the result that the yield in rat units lies somewhere between wide limits.

Preparations Nos. 97, 110, 1492, 140 Z, 150 Z, and 170 Z were made by alkaline hydrolysis; Nos. 1493, 140 Y, 150 Y, and 170 Y, by acid hydrolysis. After boiling in alcoholic solution with acid, NaOH was added to convert the fatty acids into soaps. From this point the further treatment of the soaps was identical. The alcoholic solution was diluted with water and extracted with ether. The ethereal solution was washed with distilled water to remove the alkali, then with dilute HCl to remove organic bases, next with water to remove the acid, and finally dried with anhydrous sodium sulfate. The yields obtained by the hydrolytic procedures

TABLE III.

Comparison between Degree of Purification Effected by the Four Methods of Preparation and Indicated by a Diminished Total Solid Content per Unit of Source Material.

No.	Source of preparation.	Total solids per cc. or gm. of material.			
		Routine.	Routine + acetone at Stage A.	Acid hydrolysis.	Alkali hydrolysis.
		mg.	*mg.*	*mg.*	*mg.*
176	Liquor folliculi.	3.1			
184	" "		0.83		
99 d	" "	1.00			
1492	" "			0.90	0.90
140	" "	2.44		0.64	0.92
150	" "	1.90		0.51	0.19
170	" "	3.00		0.70	0.24
169	Whole ovaries.	15.0		10.0	3.9

It is of interest to mention that there existed no difference in the rat units per kilo of the three types of preparation in each series in the case of Nos. 140, 150, 169, and 170.

are given in Table II. The maximum number of rat units by alkaline hydrolysis was 1,500 on preparation No. 110; by acidic hydrolysis 430 on No. 150 Y. Alkaline hydrolysis on the same sample of liquor folliculi yielded the same number of rat units (No. 150 Z). The relation between solids obtained in the final extract by the different processes is expressed in Table III.

Many apparent discrepancies in yield per kilo and in solids per unit may possibly be explained by variations in the original liquor folliculi. Some preliminary experiments seem to indicate that the concentration of hormone increases as the follicle matures.

In Table IV we have given the number of rat units per kilo of tissue from which it can be seen that the concentration in the liquor folliculi is greater than in the residual tissue or whole ovary. The large yields obtained from liquor folliculi in comparison with the low yields from whole ovaries or "shucked" ovaries leads us to suspect that most of the hormone is found in the liquor. Since it is impossible to remove all of the liquor by aspiration, active preparations from "shucked" ovaries are not surprising. In view of the small amount of hormone obtained from whole ovaries and especially those with only small follicles (Nos. 178 and 179) the doubtful results of previous investigators are easily understood. The use of liquor gave us a concentrated starting material which could be easily handled.

When the ratio of rat units to total solids is considered, the advantage of starting with liquor is apparent. Even when the time of aspiration is considered it is a saving of time of extraction to use the liquor. Thus far we have not made any extracts from whole ovaries approaching in purity those made from the liquor, the total solids per rat unit of the best preparation from the former being 64.0 mg. and from the latter 0.31 mg. We have, therefore, two very good reasons for the use of liquor as starting material in an attempt to isolate the hormone as a chemical individual.

A comparison of the maximum activity thus far attained of preparations from whole ovaries, placenta, and liquor folliculi is interesting. Reference to Table IV shows the maximum number of rat units obtained by extraction of liquor folliculi, whole ovaries, and human placenta to be 2,180, 160, and 700, respectively. This measurement of the physiological effect in rat units should not be construed that we believe the active constituents of the ovary and placenta are identical. Our chemical evidence on this point is incomplete since practically all of our work has been done on preparations from liquor folliculi.

Table V gives an idea of the distribution of the substance or substances producing hyperplasia in the uterus and vagina. At the outset of our work, we tested a number of different materials, the important point being whether our results were due to a hormone or a substance of non-specific character. The only tissue besides the ovary in which we have found the hormone is the pla-

TABLE IV.

Yield of Hormone from Various Tissues: Solids per Rat Unit.

No.	Tissue.	Rat units per kg. of tissue.	Total solids per rat unit.
			mg.
	Liquor folliculi—minimum yield and purity.	220	19.5
	" " maximum " " "	2,180	0.13
141	Whole ovaries.	160	64.0
102 R	" "	100	100
177	" " small follicles, non-pregnant animals.	100	280
178	Whole ovaries, small follicles, pregnant animals.	<80	
179	" " " " " "	<80	
174	Ovaries, minus liquor folliculi.	175	84
175	" " " "	125	183
189	" cystic.	200	114
159	Liquor folliculi, human, cystic.	300	
94	Placenta, human.	700	
182	" "	>400	<49.0
188	" "	580	25.0

TABLE V.

Distribution of Hormone in Various Tissues.

Source of preparations.	No. of preparations.	Results of tests.
Liquor folliculi from normal hog ovaries	85	+
" " " cystic " "	9	+
" " " human follicular cysts	4	+
Whole ovaries—cow	1	+
" " pig	16	+
Placenta—human	9	+
Corpora lutea, hog ovaries	27	−
Beef testicles	1	−
Pancreas	1	−
Hens eggs, fresh*	4	−
Fish "	4	−
Cholesterol	2	−
Embryos, pig	3	−

* One questionably positive test.

centa which physiologically seems quite logical to us and the biological significance of which will be discussed in a later publication.

The Properties of the Partially Purified Hormone.—Soluble in ether, chloroform, petroleum ether (b.p. 40–60°), alcohol (95 per cent), and acetone; insoluble in water. When freed from cholesterol it forms colloidal solutions in water. It is thermostable and fairly resistant to mild hydrolysis with dilute acids or alkalies. It is not decomposed by digestion with pancreas hash. Fairly pure preparations of the hormone are soluble in Mazola to the extent of 20 or more rat units per cc.

Although we have used Mazola as the solvent of the hormone for injection, we find its use not entirely satisfactory. Repeated injections of the oil produce granulomas and sometimes render the rats unserviceable. A more suitable menstruum is desirable.

SUMMARY.

A method of extracting a hormone from liquor folliculi which induces full estrous growth in the tissues of the genital tract is described. The yield in rat units from liquor folliculi is compared with the yield from other tissues. These results emphasize the physiological importance of the graafian follicle in the sexual cycle. A few properties of the hormone are given and data are cited to show that the activity of the follicular hormone is not associated with cholesterol color reactions.

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THE NUTRITIVE PROPERTIES OF MILK.

III. THE EFFECTIVENESS OF THE X SUBSTANCE IN PREVENTING STERILITY IN RATS ON MILK RATIONS HIGH IN FAT.*

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The failure of reproduction in rats on rations whose protein and vitamin content is supplied entirely by whole milk powder, as heretofore reported (1, 2), has since been observed by several investigators, among them Palmer and Kennedy (3) and Sure (4). It has also been confirmed recently by Anderegg (5) for milk rations such as we used. In these there exists a quantitative or qualitative deficiency which causes infertility. The search for this deficiency and the demonstration that the substance known as X (6) can supply it, constitute the subject matter of this paper.

Unwilling to postulate the existence of another accessory factor until other possibilities were exhausted we turned first to the nucleoproteins. It is generally accepted that the organism can synthesize them, and their absence in milk might even be considered as a proof of this. It seemed not impossible, however, that for the particular syntheses involved in germ cell formation some original units might be necessary. The important contributions of McCollum, Simmonds, and Parsons (7), showing the superiority of nuclein-containing proteins and the secondary quality of milk proteins, suggested that if this disparity were responsible for the lack of reproduction on a milk diet, it might be corrected by nucleoprotein supplements.

*A report of the earlier part of this paper was presented before the Western New York Branch, Society for Experimental Biology and Medicine, in April, 1923 (Mattill, H. A., and Carman, J. S., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 420).

Milk Rations With Nucleoprotein Additions.

Accordingly a series of rations was arranged, each containing 2 or 5 per cent of dried kidney or liver, or 2 per cent of thymus, or 2 per cent of yeast nucleic acid, 50 per cent of whole milk powder (Merrell-Soule), 15 per cent of lard, 2 per cent of a salt mixture (McCollum's No. 185), and 28 or 31 per cent of cornstarch. In order to verify the conclusion that a lack of vitamin B was not responsible for the inadequacy of the basal milk diet, a ration like the above but with 2 per cent of Harris' vitamin was also prepared. The kidney and liver were prepared as in McCollum's experiments (7), the thymus was kindly supplied by Parke, Davis and Co., and the other constituents were the usual market preparations. The choice of a 50 per cent level for the milk powder rested upon the observation (2) that this level was quite like 60 and 70 per cent and better than 90 to 100 per cent.

62 animals placed at weaning on these seven rations and properly mated in small cages, were observed as to growth and fertility for periods of 150 to 250 days, many of them for a longer time. None of them died; all were anesthetized for examination, five of them before 150 days. Because of the striking uniformity exhibited by all the animals, it is unnecessary to detail separately the observations made on each ration, nor is it necessary to refer to their growth beyond the remark that it was similar to that of animals on the various milk rations without additions (2), normal in males except after about 175 days, and subnormal in females after 75 to 100 days. This decline was most marked in those receiving the nucleic acid supplement. On rations containing liver and kidney the males showed exceptionally good growth, and were always above the standard curve of Donaldson, while the females declined nearer 150 days or not at all.

There was no reproduction in any of these animals. Of the thirty-three females, only one, one of four on the Harris' vitamin supplement, at 113 days of age, gave birth to a litter of five, which were dead the 2nd day. Of the remaining thirty-two females, seven (22 per cent) showed resorptions as indicated by the weight curve; in several instances autopsy revealed embryos in process of being reabsorbed. Very probably other resorptions were not detected because time did not permit of the daily examination

of vaginal smears. Some of the females from each ration were tested with males from the stock colony and of proven fertility. Of fifteen so tested, ten (67 per cent) showed resorption, but no births, thus indicating that the experimental males somehow also shared in the deficiency.

Previous attempts (2) to find a cause for the failure to bear young by weighing and sectioning the ovaries were not successful. While the weights of the ovaries were often much below the normal, so were those of fertile animals ofttimes. Nor did sections reveal any abnormality. However, in the hope of discovering some structural variation, sections of ovaries from nine of the thirty-three females were prepared, some partial, some serial, of one or both ovaries, but they indicated normal organs, with several corpora lutea and with follicles in all stages of development. Apparently ovulation was normal and the defect had, therefore, to be referred entirely to the uterus, which failed of proper implantation. This fault, however, did not account for the greater number of resorptions with fertile stock males than with experimental males.

An examination of the gonads of the experimental male animals provided the explanation. This examination consisted in weighing the testes or in the preparation of sections and smears, or both, and the observations are in accord with those made before (2).

Of the twenty-nine males only two showed normal gonads, and these were among the youngest animals examined, 109 and 139 days old. The others, ranging in age from 125 to 342 days, showed signs of degeneration. The typical signs are greatly decreased size and weight of the testes, and a dark semitransparent glassy appearance; when punctured they collapse, oozing out clear lymph; in advanced cases they may be quite shrivelled and wrinkled. In section they show a profound degeneration of the germinal epithelium. The tubules may be entirely obliterated; in less advanced cases remnants of the tails of spermatozoa are still present, but no heads can be found. Added to this is a marked proliferation of interstitial tissue together with numerous clear amorphous spaces. These changes are clear from Figs. 1 and 2. They have usually been more marked the older the animal, with great variability. Thus one animal at 324 days had testes weighing 49 per cent of the normal, and another on the same ration

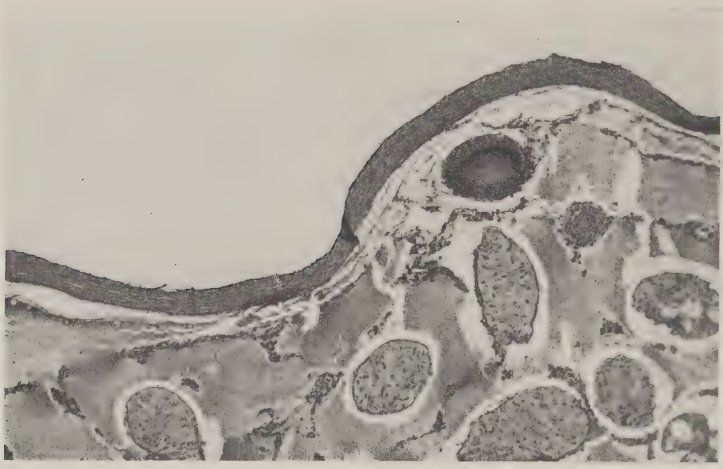

FIG. 1. Section of testis of rat at 212 days of age on a ration containing milk powder, lard, salts, and corn-starch, but none of the X substance. × 90.

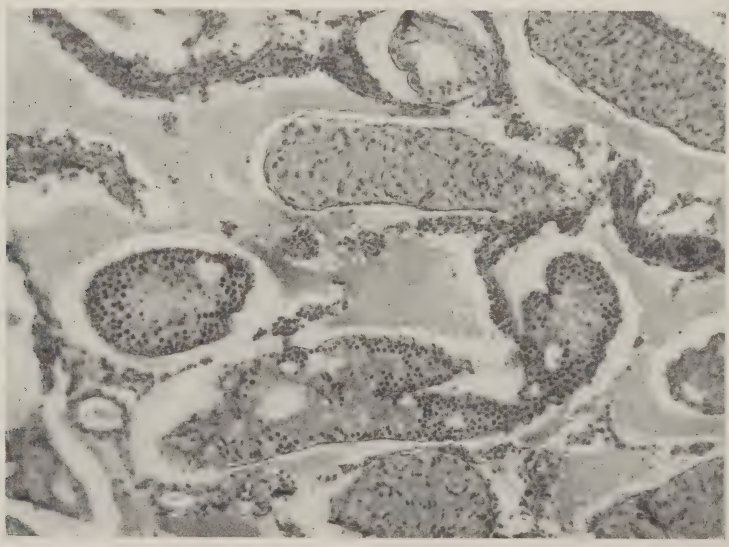

FIG. 2. Section of the same organ showing degeneration of the germinal epithelium, complete absence of sperm, proliferation of interstitial tissue, and clear amorphous areas. × 180.

at 237 days of age rated 36 per cent of the normal. Also sperm may be found, though sometimes poorly formed, in an organ fairly far below normal weight, presumably originating in tubules that have not yet suffered dissolution.

This degeneration seems to be a slowly progressive process. Six of the animals gave evidence of having once been fertile, judging from the reabsorptions of the females, but it is difficult if not impossible, on the basis of the data, to say when actual procreative power ceased. Of eight males tested with fertile females from stock, none proved fertile. The youngest was 141 days old, the oldest 275. On the other hand, one male at 205 days appeared fertile as indicated by a reabsorption in the female, and at 216 days his testes were about one-half of the normal weight. It may be questioned whether the mere presence of sperm is a criterion of fertility or whether a testis that has shrunken considerably in size may not still provide functionally active sperm from some few as yet unaffected tubules.

This progressive degeneration cannot be due to the absence of vitamin B even though it was first described as such (8) because the basal milk ration without any additions contains adequate amounts, and fully as great a degeneration occurred in the animals receiving vitamin Harris as a supplement. Nor was this degeneration to be suspected from the general appearance of the animals. There was no premature senility; on the contrary, sleek coats, silky hair, and abundant fat deposits were the rule; often the better looking the animal the more progressed was the atrophy of the testes. These facts should also dispose of the suggestion that an intoxication of intestinal origin and due to the nature of the diet was responsible. It must also be mentioned that litter mates of some of the experimental males were put on stock rations, and were used successfully for breeding purposes; when killed at 200 to 400 days of age they showed normal testes weights or slightly above. Among the many stock animals examined by weighing the testes perhaps 2 or 3 per cent have showed degeneration, and these at the age of 365 days or more.

A similar degeneration was recently described (with plates) by Eckstein (9) as appearing in rats qualitatively undernourished through lack of fat, vitamin A, and phosphorus, and suffering very marked losses in weight. It can be made to develop in an

animal that is well nourished and apparently normal in every way.

The nature of the deficiency is more obvious from the following experiments on wheat embryo supplements.

Milk Rations with Wheat Embryo Additions.

For these tests two rations were employed, one a 5 per cent, the other a 10 per cent addition of wheat embryo to the same basal ration as used above.

Of the twelve females on the 5 per cent addition ten cast litters; five of these also showed resorption. The mortality of the young was very high; in some instances the number born could not be ascertained because some had been consumed. Most of those that died succumbed within the 1st week, some during the 1st day or two. Many of these were markedly edematous and had small intramuscular hemorrhages showing through the skin. Those that survived to be continued on the ration sometimes showed unsatisfactory intestinal conditions and several of them for a time had fits of rushing around the cage at top speed until breathless. Falling prostrate with limbs extended they recovered only to begin again. From the fifteen or more litters born only twenty-three animals, among them nine females, were available for observations on the second generation.

Of these nine four were infertile or showed only resorptions, four cast litters which did not survive beyond a day or two and only one gave birth to litters of which she raised a part. Of the four third generation females which she provided three were fertile, but none of the several litters lived. Most of the new-born animals were edematous, especially in the neck region, and had hemorrhagic areas in the subcutaneous muscle layers. The cause of these disorders is unknown to us.

A consideration of this record of an unsuccessful attempt at maintaining the species leads one to ask whether, after all, natural wheat embryo does not contain substances which in certain dietary combinations, or when fed on too high a level are decidedly toxic as McCollum and coworkers demonstrated (10). In spite of this defect, however, the wheat embryo added something to the ration which caused the majority of the females in all three generations to bear young.

The potency of this constituent is particularly striking when one examines the records of the male animals. Of the eight first generation animals two had gonads below normal weight, one at 239 days 72 per cent of the normal, the other at 194 days 42 per cent of the normal. Of the fourteen second generation males five were examined after 200 days of age: four were normal or above and the fifth had one normal and one degenerated gland, the one instance of this sort we have observed. Four examined between 150 and 200 days of age were above normal. The single third generation male examined at 189 days rated 91 per cent. These figures are in marked contrast to those given in the preceding section and leave no doubt as to the existence in wheat embryo of a substance which prevents testicular degeneration on this type of ration whatever the shortcomings of the wheat germ may be.

The other group of animals on the 10 per cent wheat embryo addition contained six females; five were fertile, and cast litters repeatedly. Thus one of them gave birth to a litter on the 99th day of life and subsequently cast three more; none of these young survived beyond the 6th day. Only one of the fertile females succeeded in raising her young, but no second generation animals were long continued on this ration. The five first generation males were examined from 146 to 270 days of age and rated 90 per cent or above with one exception, 83 per cent, and this animal was functionally fertile at 212 days of age. This group of animals by its reproductive behavior emphasizes the conclusion drawn above, and it will be interesting to determine the effectiveness of smaller amounts of wheat embryo in this kind of diet.

On the assumption that the toxic and undesirable effects of wheat germ could be removed by ether extraction, the natural embryo was treated for 24 to 36 hours in a continuous extractor with anhydrous ether and then incorporated into the basal milk ration on a 5 and a 10 per cent level. To our surprise the assumption proved to be unfounded. While these tests were in progress the findings of Evans and Bishop (11, 12) came to our notice, showing that the effective material of wheat embryo resides in the oil and our results fully substantiate this conclusion. The behavior of the two lots of animals was so entirely similar that the facts may be summarized together. Of the twelve females only one at 93 days gave birth to a litter unseen. Two showed resorptions, and

the rest were entire blanks. Of the fifteen males only three were functionally fertile at 68 to 85 days of age. Twelve have thus far been examined; the testes of six of them aged 83 to 107 days assayed normal; those of the other six, aged 124 to 183 days, were 36 to 84 per cent of the normal and showed all stages of degeneration as described above. The three remaining males, having been functionally tested with several stock animals of known fertility and proved sterile, were transferred to adequate food at 150 days, but did not regain fertility during 37, 119, and 126 days, respectively. The corresponding testes weights at autopsy were 34, 39, and 39 per cent of the normal. Nine of the twelve female animals were transferred to adequate food with fertile males at 78 to 165 days of age. Six of those bore litters at 19 to 47 days after the transfer and some have continued to do so. Some of the litters quickly disappeared, others have made good growth thus indicating that lactation was satisfactory.

Tests on the Basal Milk Ration Plus Lettuce Leaves.[1]

Along with the animals thus far described, and by way of control, others were being fed the basal milk ration, and beginning at varying ages, received green lettuce leaves in undetermined amounts several times a week thereafter. The experiments are little more than qualitative because of variations in the quality and quantity of the lettuce supplied, but are nevertheless interesting in that the seven first generation females which had been infertile and had not given any signs of even resorption, all became fertile; some of the litters lived only a day or two, others survived in part and to date a few fourth generation animals have been weaned but with considerable loss in weight to the mothers. Throughout these tests an inferior lactation has been noted; the new-born animals have uniformly been normal to all appearances and those which died never showed the white streak on the abdomen indicative of successful suckling.

The observations on five male animals are also instructive. Two of them were supplied lettuce from the 156th and 180th day onward. The latter was fertile at 284 days, infertile after 325 days, and at 442 days had gonads 33 per cent of the normal weight.

[1] Mr. C. C. Congdon assisted in carrying on some of these experiments.

The animal was well nourished and exceedingly fat. The former at 255 days assayed 49 per cent with no sperm to be found. Three other animals supplied lettuce from the 92nd, 94th, and 101st days gave at 197, 192, and 249 days the following figures 91 per cent, 94 per cent, and 97 per cent. Four second generation males have given normal figures as late as 314 days.

From these data on "recovery" and the similar data in the preceding section it is apparent that male animals must be supplied with a ration adequate in the unknown substance, relatively early in life, not long after 100 days of age, if they are to retain their fertility for the expected period on such milk rations as we have employed; that female animals on the other hand may retrieve their fertility, to some extent at least, much later in life on renewal of the supply. These conclusions are perhaps to be associated with the relatively great tissue damage produced in the testes by its absence.

DISCUSSION.

The prevention of sterility on a given type of diet by the addition of another constituent can be explained only on the basis that the added constituent supplies something which is lacking in the original ration. The nature of the action of the added substance whether adjuvant, antagonistic, or detoxicating, need not be postulated. Of the substances we have tried as supplements only those which, according to Evans, are rich in vitamin X have proved successful, and we have therefore assumed as a working basis that the milk rations we have used are lacking in X.

The necessity of a substance X for reproduction has been questioned by several, especially by Nelson and his coworkers (13), who reported successful reproduction on a ration of purified foodstuffs when the fat content was reduced and with yeast as the sole source of vitamin B, and butter fat as the sole source of A. Following the same line of reasoning Anderegg (5) very recently studied milk rations with a fat content below that of ours and found satisfactory reproduction. We can confirm his observations to some extent, although our experiments have not been continued as long as his. On a diet consisting of milk powder 40, casein 8, yeast 5, salts 2, and starch, reproduction was frequent. Lactation was fairly successful, the surviving second generation grew excep-

tionally well and successfully raised a third generation that shows equally good growth; degeneration of the testes has not been found in the few animals examined at an age when it would certainly have been found on the ration higher in fat.

But these results on synthetic and milk rations low in fat hardly justify the conclusion that there is no such substance as X. If all children always had plenty of sunlight the antirachitic vitamin might not yet have been discovered; at least it would not occupy the important position in medical research that it now does. It is possible that animals on diets high in fat require more X than when the diet is poor in fat. Such a relationship would be more tangible by far than that of radiant energy to the antirachitic vitamin. Furthermore, since the distribution of X is not known except as it has been inferred from results on high fat rations, it is not impossible that foods containing amounts that are inadequate under these conditions might be entirely adequate under less demanding ones. The results of Sure (14) are of interest in this connection. If a large amount of butter fat demonstrably contains sufficient X (6) it must be present in milk and in this laboratory evidence has been obtained (2, 15) that points to its occasional presence in yeast.

Undoubtedly the ratio of fat to protein which Nelson and Anderegg emphasize is important, but the results detailed in the preceding pages are difficult of explanation on that basis.

The functions of this elusive substance in the female organism have been intensively studied by Evans; it seems to be necessary for proper implantation. The manifestations of its absence in the male rat are so different in character that one might doubt the existence of a common lack except that both disabilities are remedied by the same dietary supplement. To be sure, such a supplement might be fulfilling more than one demand. The difference in manifestation of its absence in the two sexes explains the confusing results when the combined effect is revealed in reproduction. From the data it would appear that females on these diets are never provided with adequate amounts for implantation to full term. They ovulate normally, as we have ourselves occasionally determined by the examination of vaginal smears. On renewal of the supply of X the female quickly regains her normal capacity as demonstrated by the few tests here reported and by

the extensive studies of Evans and Bishop (12). The male, on the other hand, begins his career with an abundant store of X, or else his requirement is small. At least in its absence his procreative powers are not impaired for a period of 2 or 3 months after sexual maturity. When once they do become impaired they are much more difficult to restore.

The very common failure of lactation which we have observed leads to the interesting speculation as to whether the same principle may not be a controlling factor here as in implantation. This latter process is dependent on the development of the corpus luteum. So is likewise the former. The absence of a properly constituted lipoid substance in the corpus luteum might cause the failure of one or both functions. Experiments in this direction are in preparation.

Regarding the male reproductive system and its relation to X no theories have suggested themselves. The whole problem invites investigation.

SUMMARY.

The inability of rats to reproduce on a milk diet high in fat has again been shown. This inability is not removed by supplementing such a ration with 2 or 5 per cent of nuclein-containing proteins such as liver, kidney, thymus, and yeast nucleic acid. Nor is it removed by the addition of 2 per cent of vitamin B Harris.

Both sexes are affected. In the female implantation is unsuccessful; ovulation is apparently normal. In the male there seems to be normal procreative power to about 100 days of age. Thereafter it declines and animals examined at varying ages from 125 to 150 days onward show a progressive degeneration of the testis. When this process is advanced no sperm cells exist. The lumen of the tubules has disappeared, interstitial tissue has proliferated, and there are many clear spaces filled with lymph. The organ is 30 to 60 per cent of its normal weight and has a dark and glassy appearance.

Reproductive failure does not occur on these rations if they are supplemented with 5 or 10 per cent of wheat embryo. Mortality of the young is high, suggesting the toxic properties ascribed to wheat embryo, but resorptions are much less common and degeneration of the testes does not appear. Third generation animals were obtained on the 5 per cent supplement.

Wheat embryo extracted with anhydrous ether fails to make reproduction possible. Instead there are resorptions and degeneration. The curative principle resides in the fat. Since the feeding of green lettuce leaves also prevents the infertility produced by milk diet alone, the X substance of Evans seems to be indicated as the inadequacy of this type of ration. This is the more probable because of the similarity in reproductive behavior of females on his synthetic rations and on our milk diets; females regain their procreative capacity relatively soon after the renewal of the X supply.

Male animals exhibit the lack of X in a very different way, and our experience thus far indicates that deprivation of X beyond approximately the 150th day of life probably causes irreparable damage.

On a milk diet low in fat, *i.e.* without added lard, marked reproductive failure does not appear. This fact is considered not as evidence against the existence of X, as some have held, but as an indication that the amount of X required for normal reproductive functions depends upon the nature of the diet. Until more information on this relationship and on the natural distribution of X is available generalizations are unsafe.

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STUDIES ON PENTOSE METABOLISM.

I. A COLORIMETRIC METHOD FOR THE ESTIMATION OF FURFURAL.*

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The method, presented in this paper, is a study of the conditions under which the reactions between furfural and aniline acetate may be used for the estimation of small concentrations of furfural.

De Chalmot (1) in 1893 reported the approximate furfural content of plants and leaves, utilizing the above mentioned color reaction, but no record in subsequent literature could be found in which the details of this method were elaborated to conform to modern developments in colorimetric technique. Indeed, it was not until the work of Zincke and Mühlhausen (2) in 1905 that the chemistry of this interesting reaction was fully understood.

The red color formed in the above reaction readily detects 0.00001 per cent, and 0.00004 per cent of furfural can be determined quantitatively. Experiments have also shown that the red color characteristic of the reaction between furfural and aniline acetate is not produced if the aldehyde group of the furfural molecule is destroyed, as for example by oxidation to pyromucic acid or reduction to furfural alcohol. Hence, the reaction is very valuable for quantitatively following the course of reactions involving the aldehyde group of furfural.[1]

Methyl furfural yields a light yellow color which does not introduce any appreciable error into the furfural determination.

*Presented before the Biological Section of the American Chemical Society, April, 1924.

[1] Canizarro's reaction on furfural is being studied in detail in relation to oxidizing and reducing enzymes present in tissues and body fluids.

Hydroxymethyl furfural, on account of its low color value (3) and small volatility with steam (4), does not interfere with the estimation of furfural by the aniline acetate method.

These data demonstrate that the reaction of furfural with aniline acetate is very specific for furfural, and thus may not only be of great value in studying the decomposition of pentose-containing substances, but on account of its great sensitivity, the method is applicable to the study of the furfural-producing substances occurring in the blood, body fluids, and urine.

Study of the Conditions Suitable for the Quantitative Estimation of Furfural.

Reagents used.

Furfural.—Pfanstiehl's furfural was distilled at a pressure of 20 to 30 mm. The product so obtained was colored a faint yellow and possessed a specific gravity of 1.1596 at 20.5°C.[2]

Aniline.—C.P. aniline was distilled at atmospheric pressure and preserved in darkly colored bottles. The product must be redistilled as soon as it assumes a dark red color.

Acetic Acid.—Baker and Adamson's C.P. glacial acetic acid was used. It is very important that a pure grade of acetic acid be employed, otherwise turbidity and inhibition of color development are encountered. Several samples of U. S. P. acetic acid were found to be unsatisfactory.

Standard.—The stock solution used in these experiments was prepared so that 1 cc. was equivalent to 10 mg. of furfural. This solution is stable for at least 6 months if saturated toluene water is used to dilute the furfural to the proper volume. An excess of toluene must be avoided. Great care must be taken that the pH of the solution does not become greater than 7.0.

Furfural and aniline in the presence of mineral acids develop only a very transient color. Consequently, with hydrochloric acid distillates, the mineral acid must be neutralized before the furfural can be determined. This can be accomplished by the addition of sodium hydroxide using phenolphthalein as an indicator.

[2] Handbook of chemistry and physics, Cleveland, 7th edition, 1919, gives 1.159 as the specific gravity of furfural at 20°C.

In the presence of acetic acid furfural yields a very intense red color which is sufficiently permanent to be used for quantitative measurements. Experiments show that the intensity and stability of the color are proportional to the amounts of aniline and acetic acid employed. Therefore, in order to obtain consistent results, the amounts of aniline and acetic acid must be measured accurately. Extensive experiments indicate that the most satisfactory results are obtained when 0.5 cc. of aniline and 4.0 cc. of glacial acetic acid are used for each determination. Although larger amounts of acetic acid increase the color and its stability, greater amounts than 4.0 cc. are considered unsatisfactory due to the larger volumes of final solution that would have to be employed.

Using the above outlined proportions of aniline and acetic acid it was found that the color developed to its maximum intensity in 10 minutes and that it was stable for a period of at least 40 minutes. The red color of the above reaction is very sensitive to sunlight and must be developed in diffuse light or a dark room to prevent premature fading.

In the proposed method for furfural the most accurate results are obtained when the unknown is 10 to 20 per cent above or below the color intensity of the standard furfural solution. Satisfactory results, however, may be obtained even though the intensities of the standards vary as much as 50 per cent. If it is desired to dilute either the unknown or the standard, a diluting fluid containing 40 per cent of acetic acid and 5 per cent of aniline must be used, otherwise the results will be 15 to 20 per cent too low.

That the proposed method is quantitative over a wide range of concentrations of furfural is proven by the summary of thirty experiments with furfural concentrations ranging from 0.00004 to 0.2 per cent. The average error is 2.5 per cent. In furfural concentrations between 0.0002 and 0.2 per cent the error is 0.9 per cent and in concentrations between 0.00004 and 0.0002 per cent the error is never greater than 6 per cent.

Further evidence of the accuracy of the furfural method is afforded by the average results of twelve experiments on the recovery of known amounts of pure furfural by steam distillation from 20 per cent hydrochloric acid. The amounts of furfural

distilled varied between 0.07 and 200 mg., with a recovery of 97 to 101.5 per cent.

Table I is a comparison of the colorimetric method for furfural with the gravimetric phloroglucinol (5) method and Pervier and Gortner's electrometric method (6).

TABLE I.

Comparison of Colorimetric with Phloroglucinol and Electrometric Methods.

1. Phloroglucinol Method.

Furfural.		Deviation from phloroglucinol method.
Phloroglucinol method.	Colorimetric method.	
mg.	*mg.*	*per cent*
34.7	32.9	−5.2
33.2	33.3	+0.3
54.1	55.0	+1.5
33.0	34.3	+4.0

2. Electrometric Method.

Furfural.		Deviation from electrometric method.
Electrometric Method.	Colorimetric method.	
mg.	*mg.*	*per cent*
10.14	10.00	−1.4
11.83	11.50	−2.9
12.21	11.40	−7.1
7.80	7.60	−2.6
7.97	7.60	−4.9
4.95	5.00	+1.0

These data show that the phloroglucinol and the colorimetric methods agree exceptionally well. The electrometric method has a tendency to give high values due to the fact that the end-point is not nearly so sharp as indicated by the data of Pervier and Gortner.

Method for the Determination of Furfural.

Reagents.

Acetic Acid.— C.P. glacial acetic acid was used.

Aniline.— Redistilled C.P. aniline was employed.

Diluting Fluid.—400 cc. of glacial acetic acid and 50 cc. of C.P. aniline are diluted to 1 liter with distilled water. Preserve in a darkly colored bottle.

Standards.—The stock solution contains 1 gm. of furfural diluted to 100 cc. with toluene-saturated water. 1 cc. = 10 mg. of furfural. Dilute standards are prepared as desired by dilution of the stock solution with toluene-saturated water. Generally a 1:200 and a 1:500 dilution are found to be satisfactory; *i.e.*, 1 cc. = 0.05 and 0.02 mg. of furfural, respectively. These dilute solutions remain unchanged for about 3 weeks.

Procedure.

A portion of the distillate or reaction mixture, whose furfural content is desired, is diluted so that 2 cc. contain between 0.01 and 0.05 mg. of furfural. 1 to 5 cc. of the diluted specimen, or undiluted sample, if it contains less than 0.05 mg. of furfural per cc., are pipetted into a tube calibrated at 10, 15, and 20 cc. and 1 to 2 drops of a 0.5 per cent alcoholic solution of phenolphthalein are added. Into similar tubes, containing the phenolphthalein, are pipetted 1 cc. of the 0.02 mg. and 1 cc. of the 0.05 mg. standard. Then to both unknown and standard are added, drop by drop, from a narrow bore capillary pipette, 50 per cent sodium hydroxide until a permanent pink color is obtained. If much acid is present, the tubes should be immersed in cold water, so as to prevent overheating on neutralization. There is now added to each tube exactly 0.5 cc. of aniline and 4.0 cc. of acetic acid. The contents of each tube are diluted to 10 cc. with distilled water, thoroughly mixed, and the whole series is allowed to stand in diffuse light or in a dark cupboard for 15 minutes. They are then compared, in a colorimeter, with the standard with which they match the closest. If it is necessary to dilute further than 10 cc. use the diluting fluid described under "Reagents." Colorimetric comparisons must not extend over 40 minutes.

SUMMARY.

An accurate, simple, and rapid colorimetric method is presented for the determination of furfural based on the color produced with aniline and acetic acid.

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THE RELATION OF ARGININE AND HISTIDINE TO GROWTH.* †

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Physiological literature affords comparatively little information concerning the relation of arginine and histidine to maintenance and growth. The earliest investigation dealing with the functions of these amino acids was conducted by Henriques and Hansen in 1904. They reported that the removal from predigested proteins of arginine, histidine, and lysine, by precipitation with phosphotungstic acid, yielded a mixture of amino acids which was adequate for the maintenance of positive nitrogen balances. Their conclusion was based upon a single experiment with a growing rat, and is not at all convincing. Osborne and Mendel (1914, *a*), in an effort to improve the growth curves of rats upon diets in which the nitrogen was supplied in the form of zein supplemented with lysine and tryptophane, found in a single experiment that the addition of arginine and histidine appeared to occasion a slightly more rapid increase in body weight. The addition of histidine to the diets of two rats receiving zein supplemented with tryptophane, lysine, and arginine, produced only slight differences in growth rate, which the authors did not regard as significant (1914, *b*). Abderhalden investigated

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† This communication was presented in abstract before the American Society of Biological Chemists at St. Louis, December, 1923. See Rose, W. C., and Cox, G. J., *J. Biol. Chem.*, 1924, lix, p. xiv.

‡The experimental data in this paper are taken from a thesis submitted by G. J. Cox in partial fulfillment of the requirements for the degree of Master of Science in Physiological Chemistry at the University of Illinois.

the nutritive value of arginine and histidine in nitrogen balance experiments, but his results were inconclusive.

In 1916, Ackroyd and Hopkins found that if arginine and histidine are removed from a mixture of amino acids obtained from casein by acid hydrolysis, the remaining material is inadequate for maintenance or growth. They state that if either arginine or histidine is added to the otherwise adequate diet, no loss in weight occurs, and growth may be resumed. From these results, they conclude that the two amino acids are interchangeable in metabolism, but that one or the other must be present in the diet. Furthermore, on the basis of experiments in which the allantoin excretion of rats was determined, these authors conclude, "that arginine and histidine play a special part in purine metabolism, probably constituting the raw material (or the most readily available raw material) for the synthesis of the purine ring in the animal body." When both amino acids are absent from the diet, decreases of 40 to 50 per cent in allantoin excretion are said to occur. When only one is missing, the allantoin output diminishes about 15 per cent. In a condition of malnutrition due to tryptophane deficiency, a 10 per cent fall takes place.

Soon after the above investigation, Geiling (1917) studied the nutritive value of arginine, histidine, and lysine in adult mice. He concluded that predigested casein from which the diamino acids had been removed is inadequate for maintenance, but that the addition of either arginine or histidine renders the nitrogenous portion of the ration adequate for the preservation of body weight. In this respect his findings confirm those of Ackroyd and Hopkins. In several respects, Geiling's experiments do not appear convincing to us. The protein employed was not completely hydrolyzed, and the amino acid mixture may not have been devoid of arginine or histidine following his effort to precipitate the diamino acids. As far as one may judge from the paper, delicate color tests for histidine, though available (Knoop, 1908), were not applied to the digest. Furthermore, he experienced difficulty in securing adequate food consumption by some of his animals. On the whole, an examination of his data leads one to doubt the justification for his conclusions.

The most recent investigation along this line is a paper by Abderhalden (1922) in which are reported the results of feeding

experiments with synthetic rations. According to this author, both arginine and histidine are indispensable amino acids. In a single experiment, the addition of arginine to the diet did not remedy a deficiency caused by the absence of histidine. His investigation is in many respects remarkable, but owing to the difficulties involved in the synthesis of some of the amino acids, the available material was necessarily limited, and the experiments were of short duration. His results are open to the further criticism that frequently his animals were provided with inadequate supplies of vitamins. Some of his data are difficult or impossible of interpretation in view of the obvious multiplicity of dietary deficiencies.

In the course of certain investigations involving the use of diets from which arginine and histidine had been precipitated, we were entirely unable to verify the findings of Ackroyd and Hopkins concerning the interchangeability of the two amino acids. We therefore turned our attention to this problem, the results of which are presented in this communication.

EXPERIMENTAL.

Methods.

Young rats were employed as the experimental animals. Each rat was confined in a separate cage, and the food consumption was carefully recorded. Essentially the methods of Osborne and Mendel (Ferry, 1919–20) were followed in the care of the animals. The carbohydrate portion of the food was supplied in the form of dextrin and sucrose; fat in the form of commercial lard. Osborne and Mendel's salt mixture (1919) was the source of the inorganic portion of the ration, while the nitrogenous materials were furnished in the form of either purified casein, completely hydrolyzed casein, or completely hydrolyzed casein from which arginine and histidine had been precipitated. The following procedure was employed to secure the hydrolyzed protein preparations. 2.5 kilos of casein were suspended in 25 liters of distilled water, and digested with commercial pancreatin and fresh intestinal extracts. About 10 days after the beginning of the digestion, the solution was neutralized with ammonium hydroxide. After varying intervals, fresh pancreatin was added

until, by the end of the digestion period, 800 gm. had been used. During this time extracts of sixteen pigs' intestines had also been added. From time to time, amino nitrogen estimations were made in order to follow the progress of the hydrolysis. The results showed no further change after 16 months, but in an effort to carry the hydrolysis as near completion as possible without the use of acids, the whole was diluted with an equal volume of water, fresh pancreatin added, and digestion allowed to continue for 20 months additional, making in all a period of approximately 3 years. Evidently equilibrium had been attained during the first 16 months for practically no further hydrolysis took place during subsequent months. At the end of this time, the entire fluid was heated to boiling, filtered, and evaporated to dryness at a temperature between 50° and 60°C., first in front of a fan, then in an oven, and finally in a desiccator. About 2,650 gm. of a brown hygroscopic powder remained. This had a sharp, bitter taste. Analysis of the dry material indicated that hydrolysis was approximately 78 per cent complete (*cf.* Table I).

Preliminary experiments were now made to determine the conditions for completing the hydrolysis by the use of acid. These tests indicated that treatment with 10 per cent sulfuric acid at boiling temperature for 8 hours sufficed to yield the maximum content of amino nitrogen. Therefore the entire digest was dissolved in 10 volumes of 10 per cent sulfuric acid, and kept in a boiling water bath for the time indicated. On cooling, the solution was diluted with an equal volume of water, and most of the sulfuric acid was removed with a hot solution of barium hydroxide. The barium sulfate was filtered off, and repeatedly washed with large volumes of hot distilled water. The combined filtrates and washings were divided into two equal portions, one for the preparation of the complete amino acid mixture, and the other for the arginine-histidine-free material. In the case of the former, the sulfuric acid was almost completely removed with barium hydroxide, and the final traces of acid were neutralized by the addition of a slight excess of ammonium hydroxide. The solution was then evaporated *in vacuo* until crystallization began. Further drying was accomplished in a vacuum oven, and finally in a desiccator. A brown powder with a sharp, bitter taste re-

mained.[1] The results of analyses of the dry materials before and after acid hydrolysis are presented in Table I, together with the figures for completely hydrolyzed casein according to Van Slyke (1912).

The second half of the acid-hydrolyzed digest was treated for the removal of arginine and histidine according to the method of Kossel and Kutscher (1900–01). After filtering off the silver salts of the amino acids, and removing the excess silver and barium with hydrogen sulfide and sulfuric acid, respectively, the solution, amounting to about 25 gallons, was carefully neutralized with ammonium hydroxide, evaporated to a syrup *in vacuo*, and dried to a powder as before. The resulting light yellow material was found to contain traces of histidine as indicated by

TABLE I.

Nitrogen Distribution in Casein Digest.

Group.	After digesting with enzymes for 3 yrs.	After completing hydrolysis with 10 per cent H_2SO_4.	Van Slyke's figures for casein completely hydrolyzed with acid.
	per cent total N	*per cent total N*	*per cent total N*
Ammonia nitrogen	10.20	10.06	9.4
Amino "	56.50	69.50	72.4
Imino "	30.80	19.52	16.1
Melanin "	2.50	0.92	2.1
Total nitrogen	100.00	100.00	100.00

Hunter's modification (1922) of the Knoop test (1908). In view of this fact, the entire material was again put into solution, and subjected a second time to the Kossel-Kutscher precipitation process. On drying, the amino acid mixture no longer responded

[1] In the preparation of subsequent lots of completely hydrolyzed casein, we have employed the following procedure. 1 part of casein is suspended in 10 parts of water, and digested with 0.1 part of commercial pancreatin for a period of approximately 2 months. At the end of this time, determinations of amino nitrogen usually indicate that approximately 80 per cent of the amino groups are free. The solution is then brought to boiling, filtered, treated with sufficient concentrated sulfuric acid to make a 10 per cent solution, heated on a boiling water bath for 8 hours, and finally in an autoclave at 12 to 15 pounds pressure until hydrolysis is complete. For the autoclave heating, 2 hours ordinarily are required.

to the test, and was regarded as histidine-free. We have no information concerning the degree of completeness with which arginine had been removed. Unfortunately, this amino acid does not respond to delicate color tests. Attempts to detect it by successive treatment with arginase (liver extract) and urease preparations, and the removal of ammonia from the alkaline solution with a current of air, have proven entirely unsuccessful. Both with the digest, and with hydrolyzed protein from which arginine had not been precipitated, no greater quantities of ammonia were obtained than with control samples which had not been subjected to the action of arginase. While the method is applicable to pure arginine solutions (Hunter and Morrell, 1922), it has not given satisfactory results in our hands when applied to protein hydrolysates.

The diets employed in the experimental work are summarized in Tables II and III. The term "deficient digest" is used to denote the completely hydrolyzed casein from which arginine and histidine had been precipitated. The amino acids employed to supplement the rations were prepared in this laboratory, and invariably were analyzed and found to be pure before being incorporated in the rations. Cystine was prepared by the procedure of Folin (1910), tryptophane by Dakin's modification (1918) of the Hopkins-Cole method (1901–02), histidine monochloride by the procedure of Hanke and Koessler (1920), and arginine carbonate or nitrate by the method of Kossel and Kutscher (1900–01). Tyrosine was recovered as a by-product in the preparation of tryptophane. Preliminary experiments showed that 25 mg. of commercial yeast vitamin[2] per animal per day were sufficient to permit normal growth of rats upon otherwise adequate diets. This was fed separately each day in the form of tablets composed of 7 parts of dextrin, 1 part of the vitamin powder, and 3 parts of water. The tablets were always devoured greedily as soon as presented to the animals. The fat-soluble vitamin was supplied by the inclusion of 5 per cent of cod liver oil in the rations.

Control Experiments.—In Chart I are shown the growth curves of five control animals in whose rations the nitrogen was supplied

[2] "Yeast Vitamine-Harris Powder," secured from the Harris Laboratories, Tuckahoe, N. Y.

TABLE II.

Diets of Whole Casein and of Completely Hydrolyzed Casein.

Composition.	Diet No.			
	4	7	6	12
Casein	14.7	14.7		
Completely hydrolyzed casein			14.2	14.05
Cystine	0.3	0.3	0.3	0.30
Tyrosine			0.3	0.45
Tryptophane			0.2	0.20
Dextrin	34.0	40.0	40.0	40.00
Sucrose	15.0	15.0	15.0	15.00
Lard	25.0	19.0	19.0	19.00
Cod liver oil	5.0	5.0	5.0	5.00
Salt mixture	4.0	4.0	4.0	4.00
Agar	2.0	2.0	2.0	2.00
Total	100.0	100.0	100.0	100.00

TABLE III.

Diets of "Deficient Digest," and of "Deficient Digest" Supplemented with Arginine or Histidine.

Composition.	Diet No.					
	8	11	14	20	16	22
"Deficient digest"	14.2	14.05	12.90	12.90	13.20	13.60
Cystine	0.3	0.30	0.30	0.30	0.30	0.30
Tyrosine	0.3	0.45	0.45	0.45	0.45	0.45
Tryptophane	0.2	0.20	0.20	0.20	0.20	0.20
Dextrin	40.0	40.00	40.00	40.00	40.00	40.00
Sucrose	15.0	15.00	15.00	15.00	15.00	15.00
Lard	19.0	19.00	19.00	19.00	19.00	19.00
Cod liver oil	5.0	5.00	5.00	5.00	5.00	5.00
Salt mixture	4.0	4.00	4.00	4.00	4.00	4.00
Agar	2.0	2.00	2.00	2.00	2.00	2.00
Arginine carbonate			1.29*			
" nitrate				1.40†		
Histidine monochloride					1.19‡	0.50
Total	100.0	100.00	100.14	100.25	100.34	100.05

*Equivalent to 1.13 gm. of arginine.

†Equivalent to 0.99 gm. of arginine, which is equivalent to the sum of the arginine and histidine present in casein.

‡Equivalent to 0.88 gm. of histidine, which is equivalent to the sum of the arginine and histidine present in casein.

in the form of whole (unhydrolyzed) casein. In these experiments, as in all others, the nitrogenous components of the diets (protein plus amino acids) were kept at a 15 per cent level. This is somewhat lower than the quantity (18 per cent) usually employed for such purposes. On the other hand, Osborne and Mendel (1915) have shown that much less than 18 per cent of casein permits normal growth provided cystine is added to the diet. We have, therefore, used the lower protein level, and supplemented with cystine, in order to economize in the use of the hydrolyzed casein.

The growth curves show in each case practically normal increases in body weight, except toward the ends of the experiments during the maximum heat of the summer months. Possibly the excessively hot weather may account for the lower food consumption and unsatisfactory growth of the animals at this time. The experiments are sufficiently successful for the purpose in question; namely, to prove that the diets were adequate when unhydrolyzed casein constituted the form of nitrogen.

Growth on Completely Hydrolyzed Casein.—In Chart II are recorded the growth curves of three animals upon diets in which the sole form of nitrogen was completely hydrolyzed casein. As will be observed, the rate of increase in body weight was considerably slower after the change from the whole casein to the hydrolyzed casein ration. On the other hand, the curves are more satisfactory than any hitherto reported for animals upon similar diets. That the requirements of maintenance may be covered by predigested protein has been repeatedly demonstrated. Perhaps the most successful experiments of this nature are those of Abderhalden (1915–16), who has shown that animals may maintain nitrogen equilibrium, and in some instances gain in weight, when the nitrogenous component of the ration is supplied in the form of completely hydrolyzed protein or completely hydrolyzed tissues. With less success, Mitchell (1916) endeavored to maintain adult mice upon diets containing definite mixtures of purified amino acids. In our experiments, growth rather than maintenance was the goal sought, and we regard the results as surprisingly good. Doubtless the bitter taste of certain amino acids is largely responsible for the poor food consumption in such investigations. In an attempt to mask, as far as possible, the

disagreeable flavor of hydrolyzed casein, and thus overcome the tendency of the animals to reject the food, we have always included 15 per cent of sucrose in the diet. While the curves do not indicate normal rates of body increase they nevertheless clearly show that *rats can grow for relatively long periods of time upon rations in which the nitrogenous needs of the organism are met by a mixture of amino acids alone.* We regard this fact as especially interesting and important. Its demonstration has suggested other lines of investigation, some of which we now have under way. We are also endeavoring to supplement our diets of hydrolyzed casein in such a fashion as to secure perfectly normal growth curves.

Growth upon Diets of "Deficient Digest" Plus Arginine.—In Charts III and IV are presented the results of growth experiments upon animals fed, first, diets of whole casein, then diets of hydrolyzed casein from which arginine and histidine had been removed ("deficient digest"), and third, diets of deficient digest supplemented with arginine. As will be observed, the animals increased in body weight quite rapidly on the whole casein rations, but promptly lost weight when the normal diets were replaced by those from which arginine and histidine had been precipitated. Nor did the addition of arginine carbonate to such diets improve the growth curves. Even the inclusion of 1.29 per cent of arginine carbonate, which is more than equivalent to the sum of the arginine and histidine content of casein, entirely failed to affect the loss in weight. In Rats 22 and 31 (Chart IV), we also tried the effects of arginine nitrate. It seemed scarcely possible that the form of the arginine salt could account for the differences between our results and those of Ackroyd and Hopkins. But in view of the fact that the latter investigators employed the nitrate, it seemed advisable to test its action also in our animals. The results were entirely in accord with the observations made with arginine carbonate.

It then occurred to us that the fault might be with our arginine preparations. The latter had analyzed correctly, but in order to exclude the possibility of an error in material, we tested the effect of a sample of arginine secured from an entirely different source. This was made possible through the courtesy of Prof. C. L. A. Schmidt of the University of California, who kindly

supplied us with a few grams of arginine picrolonate prepared by his electrolytic method (Foster and Schmidt, 1922). This, when transformed into the nitrate, also was found to be entirely without influence upon the growth curves. More recently we have tested several other arginine preparations, and invariably have obtained identical results. It is perfectly evident, therefore, that *arginine cannot replace histidine in the diet.*

TABLE IV.

Food Consumption and Body Weight Changes on Diets of Whole (Unhydrolyzed) Casein (Rat 11), and Completely Hydrolyzed Casein (Rat 19).

Days.	Diet No.	Average daily change in body weight.	Average daily food consumption.
	Rat 11. ♂		
		gm.	*gm.*
1–25	4	+1.08	3.5
26–60	7	+1.46	5.0
61–90	7	+1.00	5.4
91–120	7	+0.83	6.6
121–150	7	+0.77	8.0
151–180	7	+0.50	8.2
	Rat 19. ♀		
1–30	4	+1.10	4.8
31–46	6	+0.31	4.9
47–68	12	+0.55	5.2
69–98	12	+0.77	5.9
99–128	12	+0.63	5.8
129–158	12	+0.87	7.9
159–190	12	+0.31	8.0

It is of interest to note that the addition of 1.7 per cent of lysine dichloride to the diet of Rat 31 likewise failed to improve the growth of the animal.

Growth upon Diets of "Deficient Digest" Plus Histidine.—In Charts V to VIII, inclusive, are shown the effects of adding histidine to diets from which arginine and histidine had been removed. In every case there was a very sharp break in the curve, and the animal which had been losing steadily on the diet of deficient digest, rapidly increased in body weight. This was true whether the histidine was mixed with the other articles of food or, as in the case of Rat 30 (Chart VIII), was fed separately.

TABLE V.

Food Consumptiom and Body Weight Changes on Diets of "Deficient Digest" Plus Arginine.

Days.	Diet No.	Average daily change in body weight.	Average daily food consumption.
	Rat 13. ♀		
		gm.	*gm.*
1–10	4	+1.40	3.9
11–20	4	+1.50	4.1
21–28	4	+1.13	4.8
29–38	8	−1.00	1.9
39–54	11	−0.37	2.3
55–62	11 + 40 mg. arginine carbonate daily.	−0.13	2.8
63–72	14	−0.10	2.1
73–92	14	−0.25	2.0
93–112	14	−0.10	1.9
113–132	14	−0.15	2.7
133–156	14	−0.12	3.2
157–169	14	0	3.8
	Rat 20. ♀		
1–12	4	+1.50	3.8
13–24	4	+1.25	4.3
25–34	8	−0.80	2.1
35–50	11	−0.38	2.1
51–58	11 + 40 mg. arginine carbonate daily.	−0.25	2.6
59–78	14	−0.10	2.3
79–98	14	−0.25	1.9
99–106	14	0	1.9
	Rat 22. ♀		
1–10	7	+3.50	7.0
11–20	11	−1.60	2.4
21–34	11	−0.36	2.5
35–50	20	−0.25	2.5
51–68	14	−0.39	2.2
	Rat 31. ♀		
1–10	7	+4.30	9.3
11–20	11	−2.30	2.1
21–34	11	−0.36	2.4
35–50	20	−0.50	2.2

TABLE V—*Concluded.*

Days.	Diet No.	Average daily change in body weight.	Average daily food consumption.
	Rat 31. ♀—continued.		
		gm.	*gm.*
51–60	14	−0.30	1.8
61–70	14	−0.70	2.0
71–86	14	+0.06	2.9
87–98	14 + 1.7 per cent lysine dichloride.	−0.08	3.7
99–105	11	−0.14	3.9
106–115	11 + 0.05 per cent histidine monochloride.	−0.09	4.4
116–129	11 + 0.1 " " " "	+0.07	5.3
130–140	11 + 0.1 " " " " + 0.8 per cent arginine carbonate.	+0.33	5.6

In the experiments upon Rats 18, 23, and 17 (Charts V to VII), the histidine content of the food was progressively decreased following the rapid growth upon Diet 16. The latter contained 1.19 per cent of histidine monochloride (corresponding to 0.88 per cent of the free amino acid), which is equivalent to the sum of the arginine and histidine content of casein. With decreased amounts of histidine monochloride, 0.1 per cent was found to be approximately the minimal quantity which sufficed for maintenance. With this amount, growth did not occur, or at best was very slow. With 0.2 to 0.3 per cent of histidine monochloride, slow growth took place; and with 0.5 per cent, such as was used in Diet 22, growth occurred invariably at a rather rapid rate. It is of interest to note that upon diets containing the lower quantities of histidine, the animals usually attempted to compensate by ingesting more of the food. This fact will be made evident by a comparison of the figures for food consumption presented in Tables IV to VI, inclusive.

The experiment outlined in Chart VIII was conducted in a somewhat different fashion from those described above. In the case of this animal, instead of adding optimal amounts of histidine to the deficient diet, much smaller quantities were employed. It seemed possible that this method of procedure might afford a more delicate measure of the quantity of the amino acid required for maintenance. But here also 0.1 per cent of the monochloride

TABLE VI.

Food Consumption and Body Weight Changes on Diets of "Deficient Digest" Plus Histidine.

Days.	Diet No.	Average daily change in body weight.	Average daily food consumption.
	Rat 18.♂		
		gm.	*gm.*
1–24	4	+0.88	4.0
25–38	7	+1.07	4.3
39–60	11	−0.68	2.3
61–70	11 + 1.19 per cent histidine monochloride.	+2.00	5.2
71–90	16	+0.85	4.4
91–102	16	+0.50	4.3
103–114	22	−0.50	3.1
115–126	22 with histidine monochloride reduced to 0.4 per cent.	+0.42	4.1
127–132	22 with histidine monochloride reduced to 0.2 per cent.	0	5.0
133–138	22 with histidine monochloride reduced to 0.1 per cent.	+0.17	4.5
139–158	22 with histidine monochloride reduced to 0.08 per cent.	−0.10	4.9
159–179	11 + 0.1 per cent histidine monochloride.	+0.05	5.8
	Rat 23.♀		
1–10	7	+3.50	7.4
11–34	11	−0.96	2.2
35–44	16	+1.80	5.2
45–60	16	+0.88	4.1
61–70	22	+0.40	4.7
71–78	22 with histidine monochloride reduced to 0.4 per cent.	+0.25	4.5
79–84	22 with histidine monochloride reduced to 0.3 per cent.	+0.50	5.1
85–90	22 with histidine monochloride reduced to 0.2 per cent.	+0.33	5.5
91–102	22 with histidine monochloride reduced to 0.1 per cent.	+0.08	5.8
103–116	11	−0.93	4.0
117–130	11 + 0.1 per cent histidine monochloride.	+0.43	5.2
131–141	11 + 0.1 " " " " + 0.8 per cent arginine carbonate.	−0.08	5.8

TABLE VI—*Concluded.*

Days.	Diet No.	Average daily change in body weight.	Average daily food consumption.
	Rat 17. ♀		
		gm.	*gm.*
1–20	4	+0.85	4.5
21–36	7	+1.63	4.5
37–58	11	−1.00	2.5
59–68	11 + 1.19 per cent histidine monochloride.	+2.40	5.7
69–80	16	+1.75	6.5
81–100	16	+0.95	5.5
101–110	22	+0.30	4.7
111–116	22 with histidine monochloride reduced to 0.4 per cent.	+0.33	5.8
117–124	22 with histidine monochloride reduced to 0.3 per cent.	+0.25	5.6
125–130	22 with histidine monochloride reduced to 0.2 per cent.	+0.50	6.2
131–140	22 with histidine monochloride reduced to 0.1 per cent.	−0.10	6.6
141–155	11	−1.25	4.6
156–190	11 + 0.1 per cent histidine monochloride.	+0.24	6.6
	Rat 30. ♂		
1–10	7	+5.00	10.0
11–32	11	−1.41	2.4
33–42	11 + 60 mg. histidine monochloride fed separately daily.	+1.40	4.1
43–62	11	−0.85	2.3
63–86	11	−0.21	3.2
87–98	11 + 1.7 per cent lysine dichloride.	−0.25	4.1
99–116	11 + 0.05 per cent histidine monochloride.	−0.11	4.4
117–130	11 + 0.10 " " " "	+0.64	5.6
131–137	11 + 0.10 " " " " +0.8 per cent arginine carbonate.	−0.14	5.3

was found to be just about sufficient to prevent loss in weight. The addition of 0.8 per cent of arginine carbonate to the diets of animals on the minimal maintenance allowance of histidine (*cf.* Charts VI and VIII) was as ineffective as when the latter was entirely excluded from the ration.

In Chart IX, are reproduced in close proximity to each other the growth curves of Rats 13 and 17. These animals, while not of the same litter, differed in age by 1 day only, and were upon diets identical in every respect with the exception of the arginine and histidine content. The contrast in growth is well exemplified. It is evident that arginine and histidine are quite independent of each other in metabolic effects. At the points

Fig. 1. The upper photograph represents Rat 17; the lower, Rat 13. The former was on a diet of deficient digest + histidine, while the latter was on a similar diet except that histidine was replaced by arginine. The growth curves of the animals are reproduced in Chart IX. The letter *"P"* indicates on each curve the point at which the photograph was taken. The time was not the most favorable one for Rat 17, inasmuch as this animal had been on a diet of deficient digest for several days preceding, and as shown in the curve, had lost about 17 gm. from its maximum weight. Despite this fact, the differences in nutritive condition of the two rats are quite striking.

marked "*P*" on Chart IX, photographs were made of the animals. These are reproduced in Fig. 1. The differences in size and nutritive condition are very striking. The posture of Rat 13 is characteristic, and has been observed in other animals upon diets devoid of histidine.

We are entirely unable to reconcile our results with those of Ackroyd and Hopkins. We hesitate to suggest that their arginine preparation may have been contaminated with histidine, but only with the aid of such an assumption can we understand their data. Whatever may be the explanation of their findings, the experiments herein described provide convincing proof that *arginine and histidine are not interchangeable in metabolism, and that histidine is an indispensable amino acid.*

Our results must not be interpreted as indicating that arginine is an unnecessary component of the diet. Indeed, the data have no bearing upon this question. It is quite likely that even after two precipitations with silver sulfate and barium hydroxide, our digests may have contained sufficient amounts of arginine to meet the needs of the animals. The silver salt of arginine is more soluble than is the similar salt of histidine. Thus appreciable traces of arginine certainly remained in solution. We expect to investigate this phase of the problem at a later date.

CONCLUSIONS.

1. Comparative studies have been made of the growth of rats upon diets in which the nitrogen was supplied respectively by casein, completely hydrolyzed casein, and hydrolyzed casein from which arginine and histidine had been removed by double precipitation with silver sulfate and barium hydroxide. Rats upon completely hydrolyzed casein grow to maturity, but at slower rates than animals of the same age upon whole casein. Rats upon the arginine-histidine-free amino acid mixture are neither able to grow nor to maintain body weight, but promptly and continually lose weight. The addition of histidine to such a ration results invariably in an immediate resumption of growth at a normal rate. *Histidine is thus shown to be an indispensable component of the diet.*

2. Experiments designed to determine the minimum histidine requirement show that the addition of 0.1 gm. of histidine mono-

chloride per 100 gm. of food (corresponding to approximately 0.5 per cent of the protein when the monochloride is expressed as free histidine) usually suffices for maintenance. 0.2 to 0.3 gm. of histidine monochloride per 100 gm. of food permits moderate growth, while 0.5 gm. (equivalent to 2.5 per cent of the protein, the proportion of histidine normally present in casein) occasions a practically normal rate of increase in body weight.

3. In contrast to the behavior of histidine, the addition of arginine to the deficient diet exerts no perceptible influence upon growth, even when the quantity added is more than equivalent to the sum of the arginine and histidine present in native casein. The animals continue to lose weight as rapidly as before the addition of the amino acid. Nor can growth be induced by arginine in animals upon the minimum maintenance allowance of 0.1 per cent of histidine monochloride. It is evident, therefore, that contrary to the observations of Ackroyd and Hopkins, *arginine and histidine are not mutually interchangeable in metabolism.* The experiments have no bearing upon the question of the indispensability of arginine, since it is quite likely that the Kossel-Kutscher method of precipitation does not remove arginine as completely as it does histidine.

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DESCRIPTION OF THE CHARTS.

Charts I and II represent the growth of rats upon whole (unhydrolyzed) casein, and completely hydrolyzed casein, respectively, and are self-explanatory. Charts III and IV show that the addition of arginine to rations in which the nitrogeneous component is supplied in the form of completely hydrolyzed casein from which arginine and histidine had been removed, entirely fails to improve the growth curves. Charts V to IX, inclusive, demonstrate the remarkable growth-promoting effect of adding histidine to the food of animals receiving the deficient digest.

In Charts III to VIII inclusive, the broken line portions of the curves represent the growth of the animals during the preliminary periods upon whole casein diets. Growth upon all other dietaries is represented by solid line curves. The vertical broken lines indicate the points where the rations were changed. In certain of the experiments (Charts IV to VII, inclusive), involving frequent alterations in the histidine content of the food, arrows, instead of vertical lines, are employed to mark the exact positions on the curves at which the changes were instituted. Concise information concerning the duration of each dietary is presented in Tables IV, V, and VI.

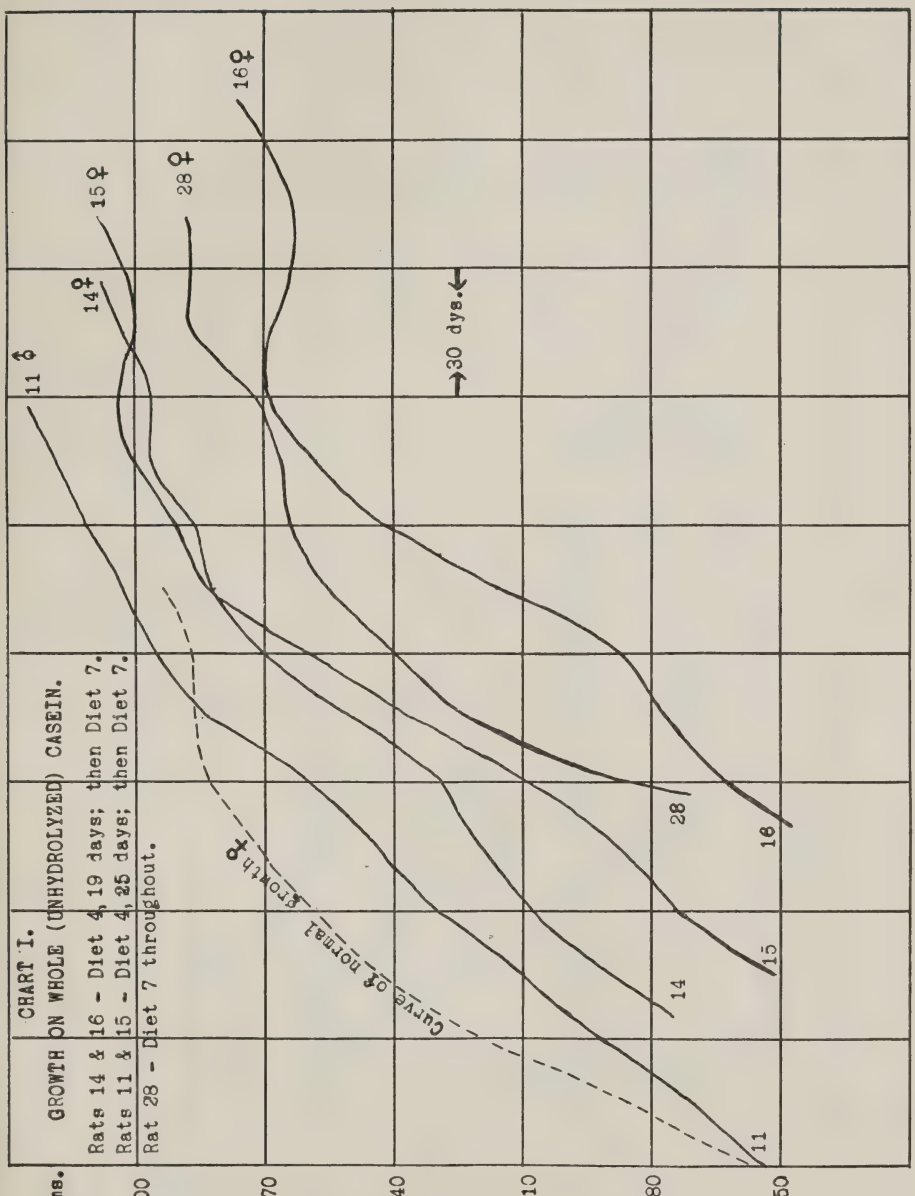
CHART I.
GROWTH ON WHOLE (UNHYDROLYZED) CASEIN.
Rats 14 & 16 - Diet 4, 19 days; then Diet 7.
Rats 11 & 15 - Diet 4, 25 days; then Diet 7.
Rat 28 - Diet 7 throughout.
Gms.
200
170
140
110
80
50
11 ♂
14♀
15♀
28♀
16♀
30 dys.
Curve of normal ♂ growth
11
14
15
28
16

Chart I.

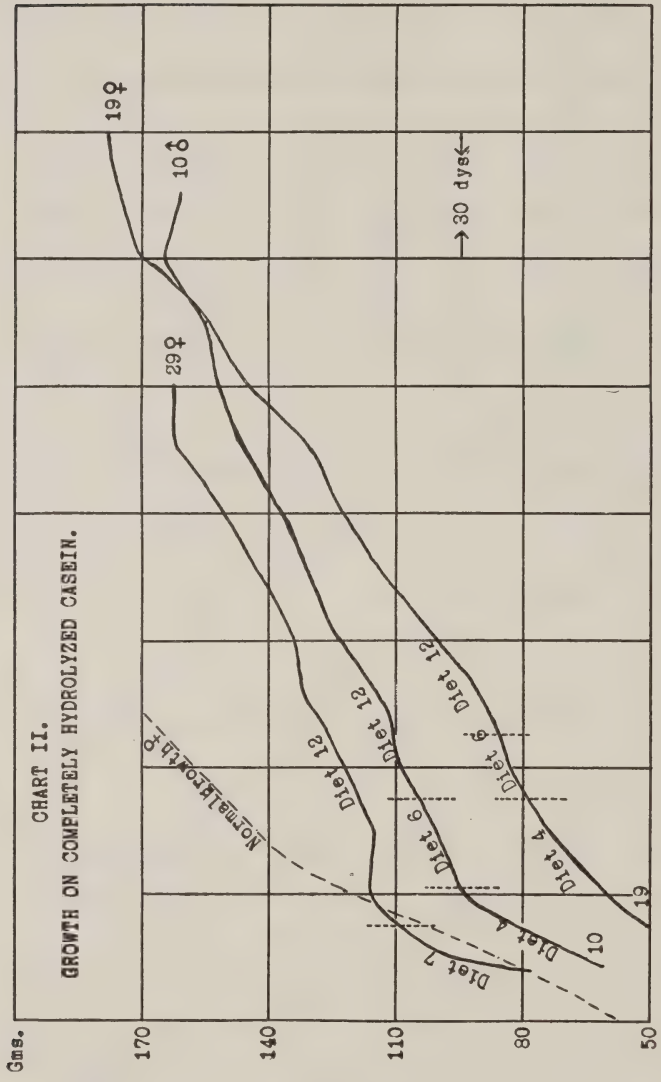
CHART II.
GROWTH ON COMPLETELY HYDROLYZED CASEIN.
Gms.
170
140
110
80
50
19♀
10♂
29♀
30 dys
Normal growth ♀
Diet 12
Diet 12
Diet 12
Diet 6
Diet 6
Diet 4
Diet 4
Diet 7
19
10

CHART II.

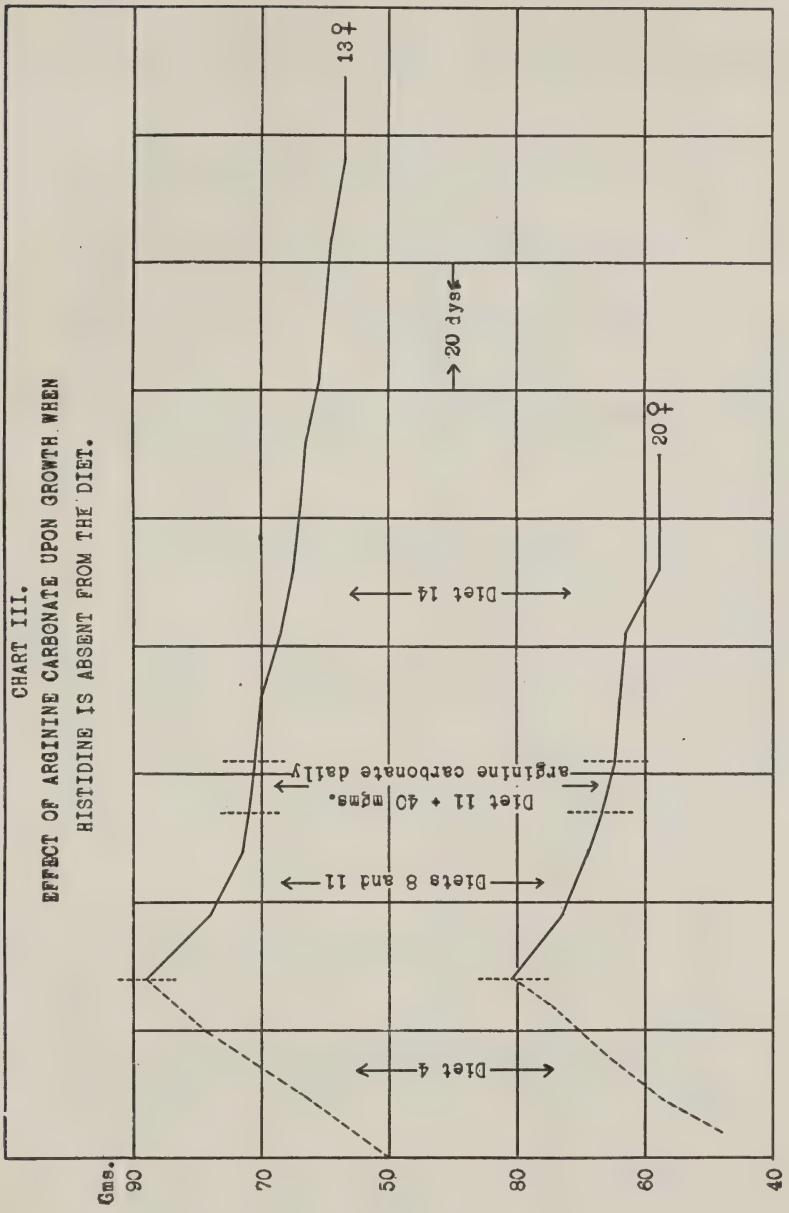
CHART III.
EFFECT OF ARGININE CARBONATE UPON GROWTH WHEN HISTIDINE IS ABSENT FROM THE DIET.
Gms.
90
70
50
80
60
40
13 ♀
20 ♀
20 days
Diet 14
Diet 11 + 40 mgms. arginine carbonate daily
Diets 8 and 11
Diet 4

CHART III.

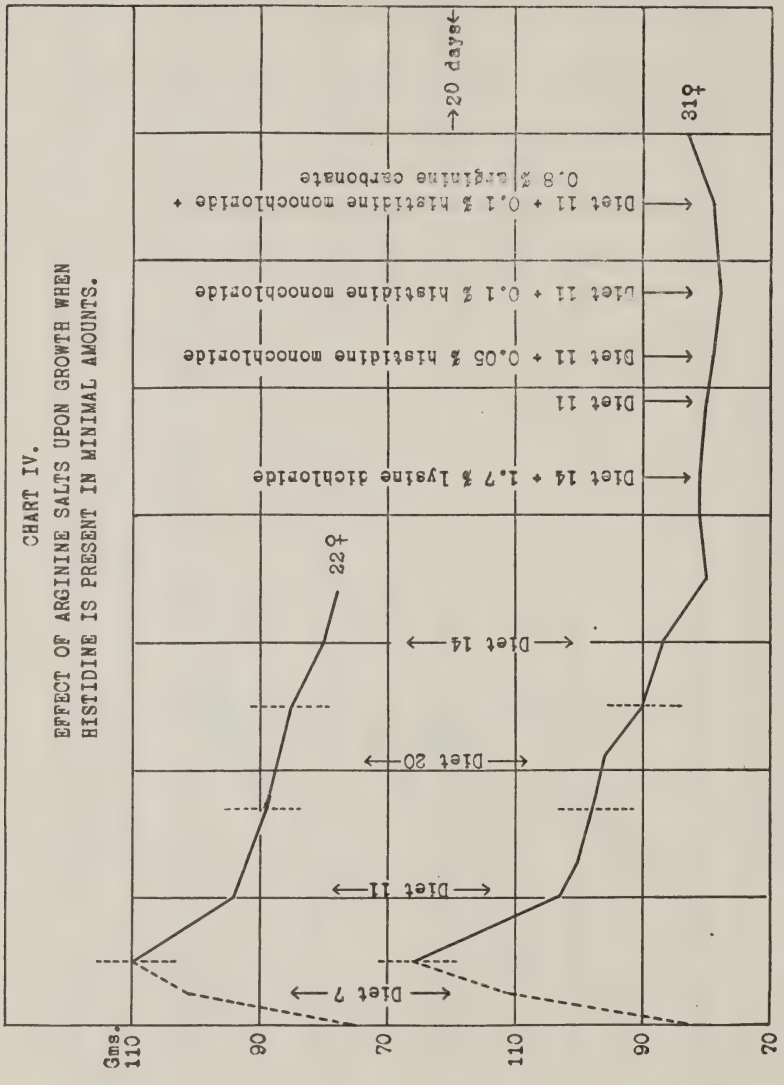
CHART IV.
EFFECT OF ARGININE SALTS UPON GROWTH WHEN HISTIDINE IS PRESENT IN MINIMAL AMOUNTS.
Gms.
110
90
70
110
90
70
Diet 7
Diet 11
Diet 20
Diet 14
22 ♀
31 ♀
20 days
Diet 14 + 1.7 % lysine dichloride
Diet 11
Diet 11 + 0.05 % histidine monochloride
Diet 11 + 0.1 % histidine monochloride
Diet 11 + 0.1 % histidine monochloride + 0.8 % arginine carbonate

Chart IV.

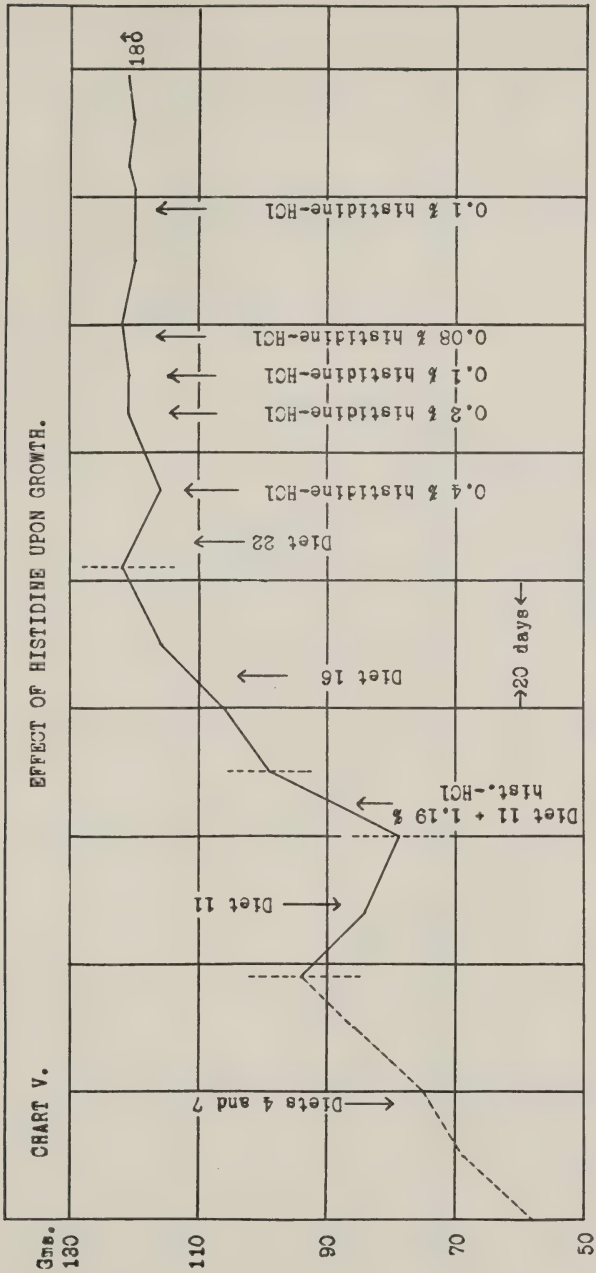
CHART V.
EFFECT OF HISTIDINE UPON GROWTH.
Gms.
180
110
90
70
50
Diets 4 and 7
Diet 11
Diet 11 + 1.19 % hist.-HCl
Diet 16
20 days
Diet 22
0.4 % histidine-HCl
0.3 % histidine-HCl
0.1 % histidine-HCl
0.08 % histidine-HCl
0.1 % histidine-HCl
180

CHART V.

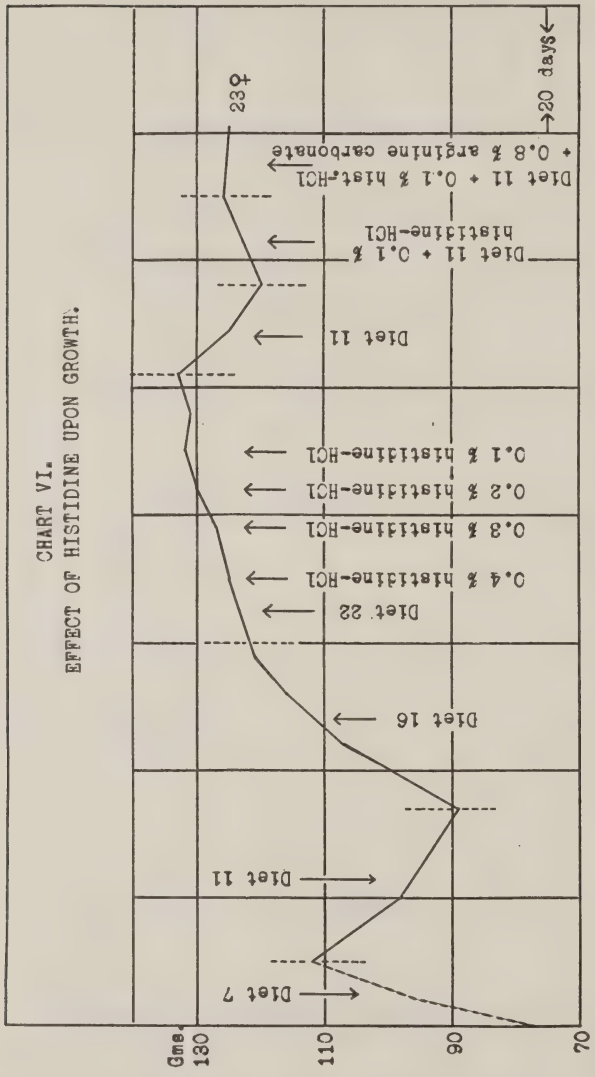
CHART VI.
EFFECT OF HISTIDINE UPON GROWTH.
Gms.
130
110
90
70
Diet 7
Diet 11
Diet 16
Diet 22
0.4 % histidine-HCl
0.3 % histidine-HCl
0.2 % histidine-HCl
0.1 % histidine-HCl
Diet 11
Diet 11 + 0.1 % histidine-HCl
Diet 11 + 0.1 % hist.-HCl + 0.8 % arginine carbonate
23♀
20 days

Chart VI.

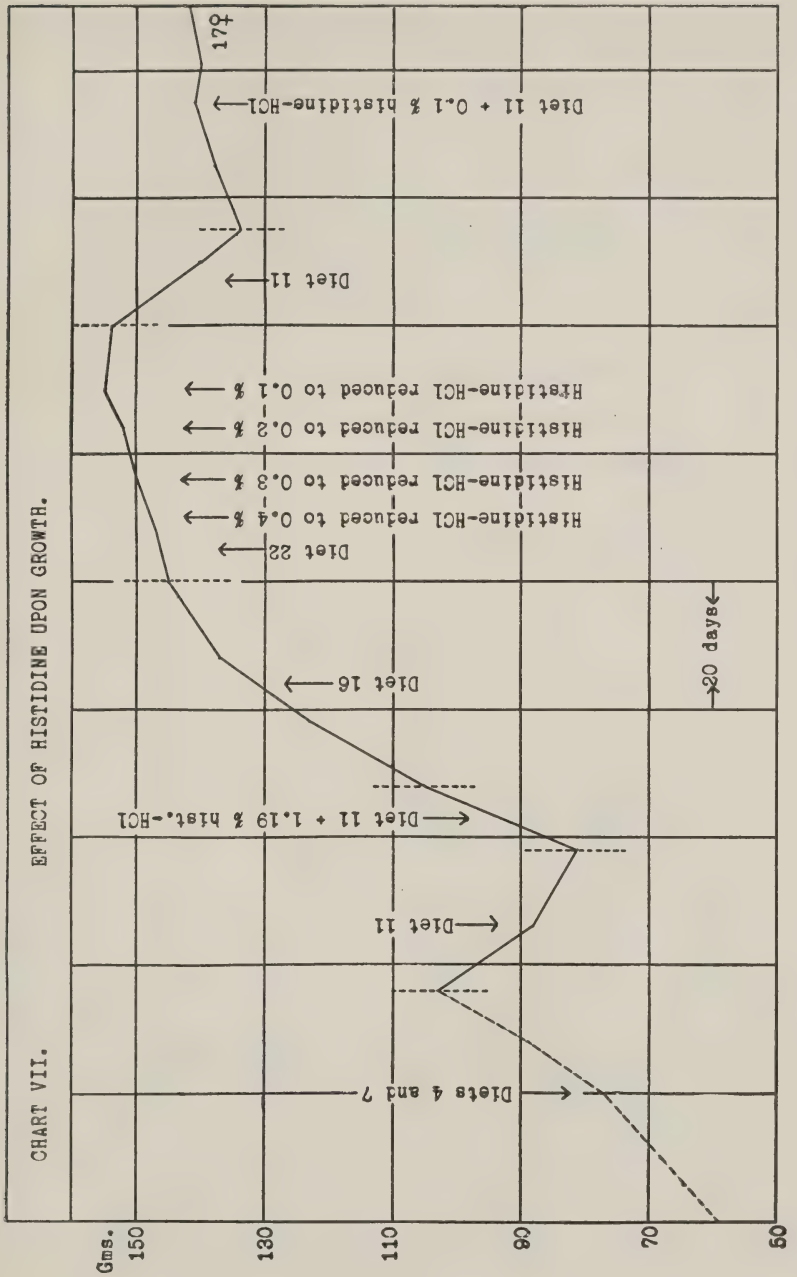
CHART VII.
EFFECT OF HISTIDINE UPON GROWTH.
Gms.
150
130
110
90
70
50
Diets 4 and 7
Diet 11
Diet 11 + 1.19 % hist.-HCl
Diet 16
20 days
Diet 22
Histidine-HCl reduced to 0.4 %
Histidine-HCl reduced to 0.3 %
Histidine-HCl reduced to 0.2 %
Histidine-HCl reduced to 0.1 %
Diet 11
Diet 11 + 0.1 % histidine-HCl
17½

CHART VII.

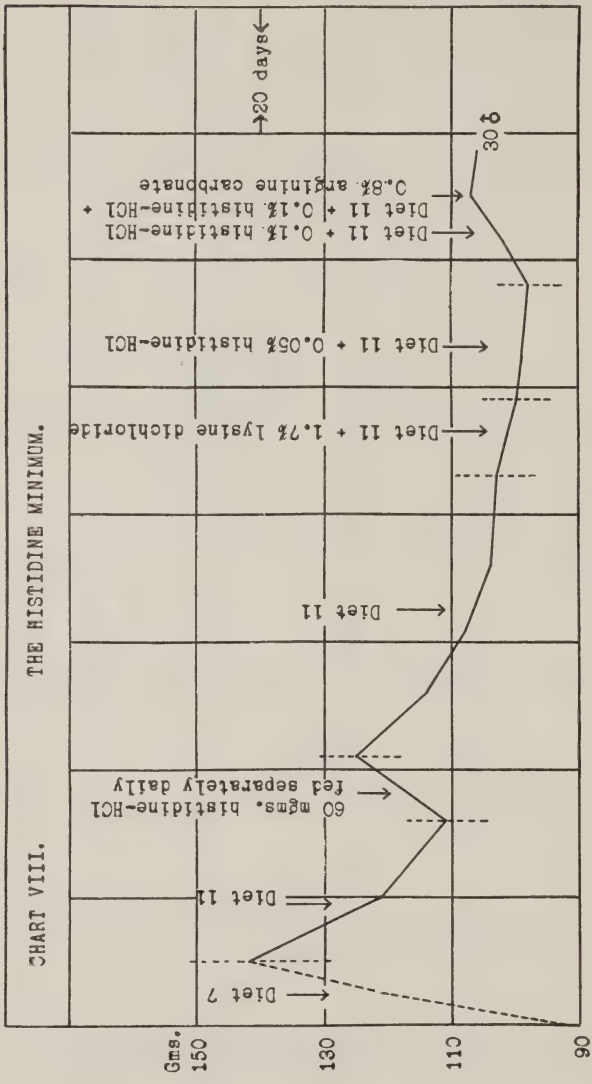
CHART VIII.
THE HISTIDINE MINIMUM.
Gms.
150
130
110
90
Diet 7
Diet 11
60 mgms. histidine-HCl
fed separately daily
Diet 11
Diet 11 + 1.7% lysine dichloride
Diet 11 + 0.05% histidine-HCl
Diet 11 + 0.1% histidine-HCl
Diet 11 + 0.1% histidine-HCl +
0.8% arginine carbonate
30 δ
20 days

CHART VIII.

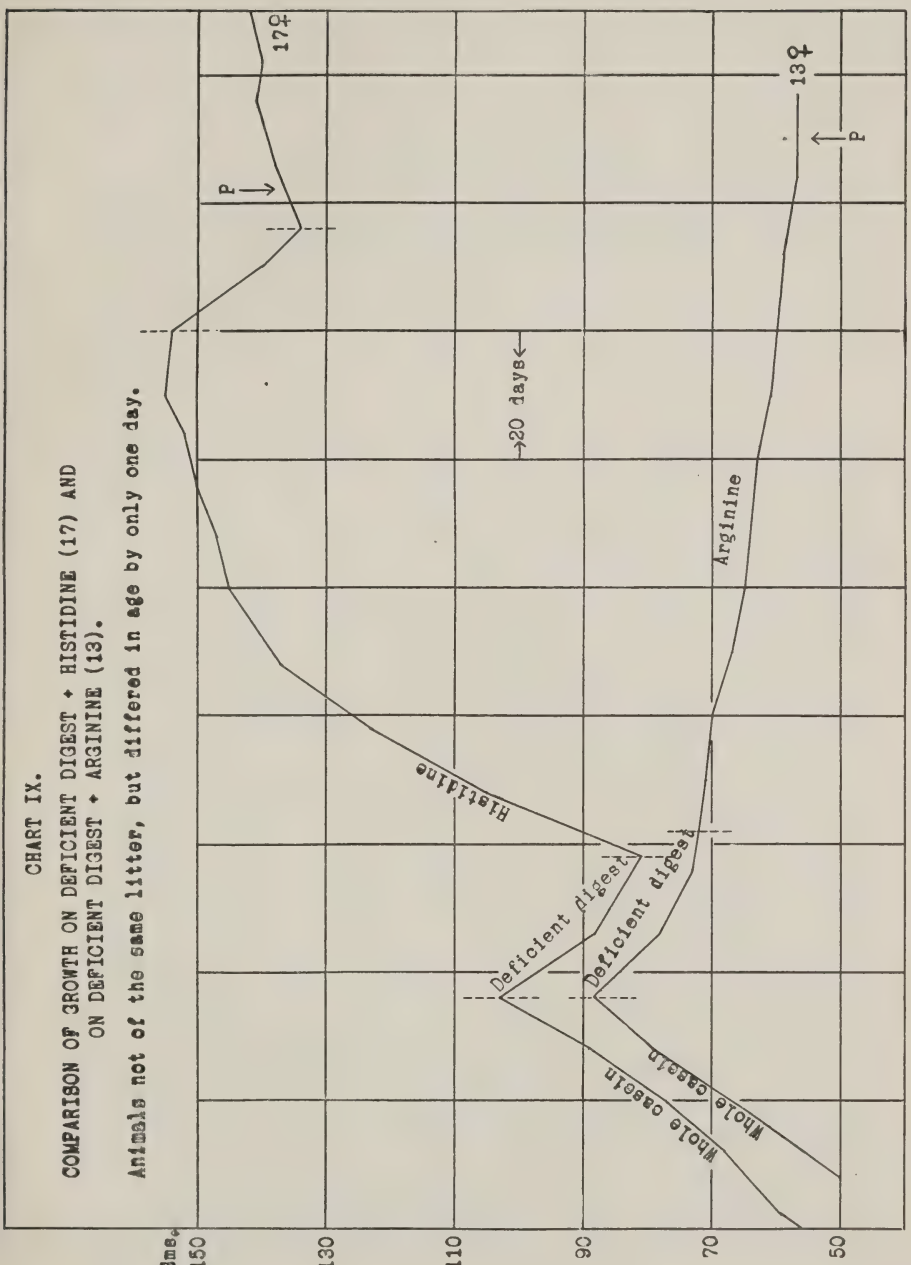
CHART IX.
COMPARISON OF GROWTH ON DEFICIENT DIGEST + HISTIDINE (17) AND ON DEFICIENT DIGEST + ARGININE (13).
Animals not of the same litter, but differed in age by only one day.
Gms.
150
130
110
90
70
50
17♀
P
13♀
P
20 days
Histidine
Arginine
Deficient digest
Deficient digest
Whole casein
Whole casein

Chart IX.

FAT-SOLUBLE VITAMINS.

XVIII. SUNLIGHT IN ITS RELATION TO PORK PRODUCTION ON CERTAIN RESTRICTED RATIONS.*†

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(Received for publication, July 26, 1924.)

There has prevailed an opinion among practical stockmen that farm animals require a certain amount of direct sunlight for physiological well-being. This undoubtedly had its origin in the observation that stock turned out in the paddock in spring, after the close confinement of the winter months, becomes more contented and improved in appearance as it basks in the solar rays. To the poultryman, especially,—always on the alert to apprehend the elusive factors concerned with egg production and the rearing of chicks, has been evident the promptness with which chickens seek out the sunlight and the resultant evidences of contentment, usually followed by reddening of the combs and wattles, smoothing of the feathers, and increased egg production. While the bactericidal action of light has long been recognized in farm practice as something worthy of recognition, as shown by the tendency of the farm architect to provide plenty of window lights for barns, generally the beneficial action of sunlight has been attributed to heat rather than to light; and the beneficial action of the range,—in the absence of change of ration, has been associated with the opportunity for exercise with resultant increased appetite and increased consumption of food.

In human medicine there has likewise been an extreme reluctance, even in the face of many definite observations, to attribute

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any beneficent physiological properties to light. While life at high altitudes has long been adjudged beneficial in certain diseases such as tuberculosis and rickets, the benefits have long been attributed to the purity of the air, its freedom from dust, its low oxygen content, its stimulating temperature, its low humidity, and its content of ozone. Only as the result of the enthusiasm of those interested in heliotherapy has light finally received the recognition that it deserves.

Much has been done by Finsen (1) at his Institute at Copenhagen especially organized to study the effect of light. He was very successful in curing lupus by means of sunlight or light from an electric arc. Rollier likewise achieved phenomenal success at Leysin at a high altitude, using sunlight in treatment for joint and bone tuberculosis (2). In 1919 Huldschinsky (3), knowing from clinical experience that rickets in children would yield to treatment with baths and exposure to air and light, showed conclusively that light is the important factor. By means of the quartz mercury vapor lamp he cured four very severe cases of rickets in children $2\frac{1}{4}$ to $4\frac{1}{4}$ years old. Since then Hess and Unger and others (4) in this country have verified the reported therapeutic value of light in clinical as well as in experimental rickets.

In the last few years with the attention that has been given to the factors operative in making possible normal growth in young animals, evidence has been accumulated that either light or the antirachitic vitamin is required for growth. Hume (5) first showed that growth in rats could be prolonged for a considerable period of time by radiation with ultra-violet light. It was believed that light substituted for vitamin A and that, therefore, vitamin A and the antirachitic vitamin were identical. Steenbock and Nelson (6), however, interpreted Hume's data, as well as similar data of their own, in a different manner. Taking into consideration the fact that the animal can store fat-soluble vitamins they concluded that ultra-violet light can substitute for the antirachitic vitamin, but not for vitamin A, and that in spite of radiation, growth finally ceases due to exhaustion of the stored reserves of vitamin A.

These findings brought out the possibility that in the absence of the antirachitic vitamin, light may be an important factor entering into the production of normal growth and well-being of many of our domestic animals. Accordingly, experiments were started by the authors with chickens and with pigs. Results obtained with the former have already been reported (7); results with the latter form the subject of the present communication.

Rickets or a condition approximating rickets has been known by practical stockmen for a good many years as occurring in pigs fed grain rations especially during the winter months. Its association with the winter season with cold and dampness has led it to be often spoken of as rheuma-

tism. Elliot and coworkers (8) are authority for the statement that 50 per cent of the pigs of certain districts born in early winter grow a rough coat of bristles, become lethargic, increase in weight slowly, and ultimately become unable to walk due to stiffness of the legs. This is the same difficulty experienced by Hart, Miller, and McCollum (9) when attempts were made to raise pigs in confinement on grain rations. Histological evidence of degeneration of the motor neurons of the spinal system was obtained which with the motor disturbances were associated etiologically with what reads now, with our present knowledge of nutrition, like a generous complex of deficiencies. Alfalfa hay incorporated in the ration at a 20 to 25 per cent level and meat scraps were found to prevent this difficulty. Since these results were published numerous other experiments have been carried out attacking the problem particularly from the point of view, that inasmuch as pigs on the range do not become afflicted, they must supplement their dietary requirements in some way or another. Particular attention was paid to the roughage factor and the probable rôle of salts. Supplements such as salt mixtures, earth, paper, charcoal, and clover and alfalfa were fed at various times, but consistent results were obtained only with the latter two. In later years with the emphasis placed upon cod liver oil as an antirachitic in human medicine, and a surmisal that the difficulty observed in these pigs might be rickets, cod liver oil was fed both as a prophylactic and curative with excellent results (10).

Zilva, Golding, Drummond, and Coward (11) studied this condition from the standpoint of vitamin A deficiency. Feeding pigs, from the time of birth on rations, respectively high and low in vitamin A, they obtained no conclusive evidence of the production of rickets. Later these same workers (12) limited calcium as well as vitamin A in the ration of their pigs. They observed osteoporosis and defective calcification in the zone of provisional calcification. Rickets in the true sense of the word was not produced which they say was probably due to too much calcium, but their controls also were not entirely normal. Elliot, Crichton, and Orr (8) in their experiments apparently did produce rickets. They report an irregular epiphyseal line, with excess osteoid and disorganization in the proliferative cartilage zone. Addition of salts reduced the rickets and cod liver oil prevented it.

In all of these experiments no mention is made of the conditions under which the animals were confined so the reader is unable to judge whether animals were exposed to light or not. Golding, Zilva, Drummond, and Coward (12) speak of light as "an hypothetical factor," but the statement of Elliot, Crichton, and Orr (8) with respect to incidence of the malady in pigs born in early winter appears significant. In view of the results obtained with light in human medicine and with laboratory animals the rôle of light in pork production under practical conditions appeared to merit investigation.

EXPERIMENTAL.

In view of the fact that the beneficial effect of light in rickets and allied conditions becomes evident only in the absence of a sufficiency of the antirachitic vitamin, it was necessary, in the first place, to select young pigs which had not had an opportunity to store much of this vitamin and in the second place to feed them a ration well balanced in its composition except for this vitamin.

In our selection of pigs we did not have wide latitude. Our experimental needs had not been anticipated, but by the courtesy of Professor Morrison of the Animal Husbandry Department we were able to secure twenty-four young pigs (reds and blacks)[1] weighing from 35 to 65 pounds and not less than 6 weeks of age. They varied considerably in weight and were older and larger than desirable, both facts being of considerable importance from the standpoint of the experiment when the pigs have been raised on a diet rich in the fat-soluble vitamins. Steenbock, Sell, and Nelson (13) have shown that a difference of a few days and 5 or 10 gm. in weight with rats on such a diet may make a difference of many weeks in growth when put on a diet deficient in these vitamins. In the minds of the writers there remains no question but that most of the results obtained in feeding experiments with swine as well as with other animals aiming to test out the efficiency of various rations with respect to content of fat-soluble vitamins have been grossly distorted by a lack of knowledge of these factors. This has already been pointed out (13) in connection with the difficulty experienced by investigators when an attempt was made to substantiate the findings of Steenbock and Boutwell (14) that yellow corn is richer in fat-soluble vitamins than white corn. Unfortunately, no accurate information on the vitamin content of the ration on which our pigs had been reared was available. This made it questionable to us in the beginning just how much in the way of accurate data could be obtained with our animals; but as no other course was available it was decided to carry out the experimental plans, hoping to obtain information on some if not all the points desired.

[1] Pigs numbered below 500 were Duroc-Jerseys; the others were Poland-Chinas.

In the light of past experience with the great variation observed in the incidence of "rickets" on various grain rations it appeared desirable to restrict the ingredients of the rations fed to these pigs as much as possible to those of known limited vitamin content, but otherwise of good growth-sustaining properties. Such a ration was constructed from yellow corn 97, sodium chloride 1, and calcium carbonate 2 with an allowance of 4 pounds of centrifugated skimmed milk per pig per day. For comparative purposes, as white corn contains less vitamin than yellow corn, a ration containing white in place of the yellow corn was also used for part of the series.

The pigs were divided into four groups, six in each, twelve being fed the yellow corn ration and twelve the white corn. On each ration, six pigs were kept in the "dark" and six in the "light." They were confined in indoor pens measuring 9 by $11\frac{1}{2}$ feet with outdoor runways of $11\frac{1}{2}$ by 12 feet. For the pigs kept in the "dark" these, however, could hardly be considered outdoor runways because they were enclosed with ship lap on 2 by 4 studding and roofed over with composition roofing at a heighth of 8 to $9\frac{1}{2}$ feet as a "lean to" so that no direct rays of the sun could penetrate at any place and diffuse reflected light was visible in but few places. Ventilation and drainage were provided for by hooded openings at the top and bottom. Both inside pens and runways were cement-floored, but in each pen a planked sleeping platform was provided. The inside pens were in no case to be considered dark in the strict sense of the word; they were, however, well shielded from any direct rays of the sun and all groups inside received the diffuse reflected illumination common to the barn, except where the runways were covered; there light entering from the doors and windows was also shut out by the "lean to." The terms "dark" and "light" are, therefore, to be considered relative only and are used for convenience.

The experiment was started June 27, 1923, and continued until January 10, 1924. This is mentioned because as is well known and as has been pointed out again by Dorno (15) recently the intensity of ultra-violet solar radiation varies decidedly with the season of the year, the variations with seasons being even greater for ultra-violet than for heat and visible light. He has reported an approximate 6-fold decrease for midday in January as compared

with July. This decrease in ultra-violet radiation is of particular significance to us in our experiment, because as is well known it can be correlated with the seasonal incidence of rickets in children. In our experiment the reduction in intensity of illumination was no doubt greater because of the reduction in time of exposure to sunlight. Our experimental pens were located directly west and north of two wings of the hog barn so that direct exposure to sunlight was possible only as the sun approached the zenith and as it came in position due west. In late fall and winter, therefore, the pigs received very little sunlight; those on the yellow corn were particularly unfortunate in this respect because their pen was nearest the barn.

Observations.

As a criterion of the progress of the experiment the animals were weighed regularly every 2 weeks. Later on blood samples were taken at intervals for phosphorus determinations and ultimately with the termination of the experiment, shaft bones and costochondral junctions were dissected out for inorganic analysis and for histological examination, respectively.

Growth.

The results on growth are shown in Charts I and II. Immediately evident, irrespective of ration or treatment, is the gross irregularity in the growth of different animals. Such results are always exceedingly disconcerting to the experimentalist as indicating that all conditions have not been absolutely under his control and in the absence of definite information are usually attributed to lack of vitality or a lack of growth impulse possessed by the animal. Such explanations, however, give little satisfaction, and to the authors at least, there has appealed more and more the justification to associate these variations in growth with differences in the stored reserves of indispensable dietary substances, —provided, of course, that contagious diseases are eliminated. This conception has been the result especially of observations made on dogs and rats. In both it was found impossible to produce rickets by lack of the vitamin or lack of light during the experimental period if the animals were not prevented from stocking up their reserves either by being kept on the mother's ration

too long or by being fed a ration too rich in the vitamin over a much shorter period of time. On this basis in view of absence of

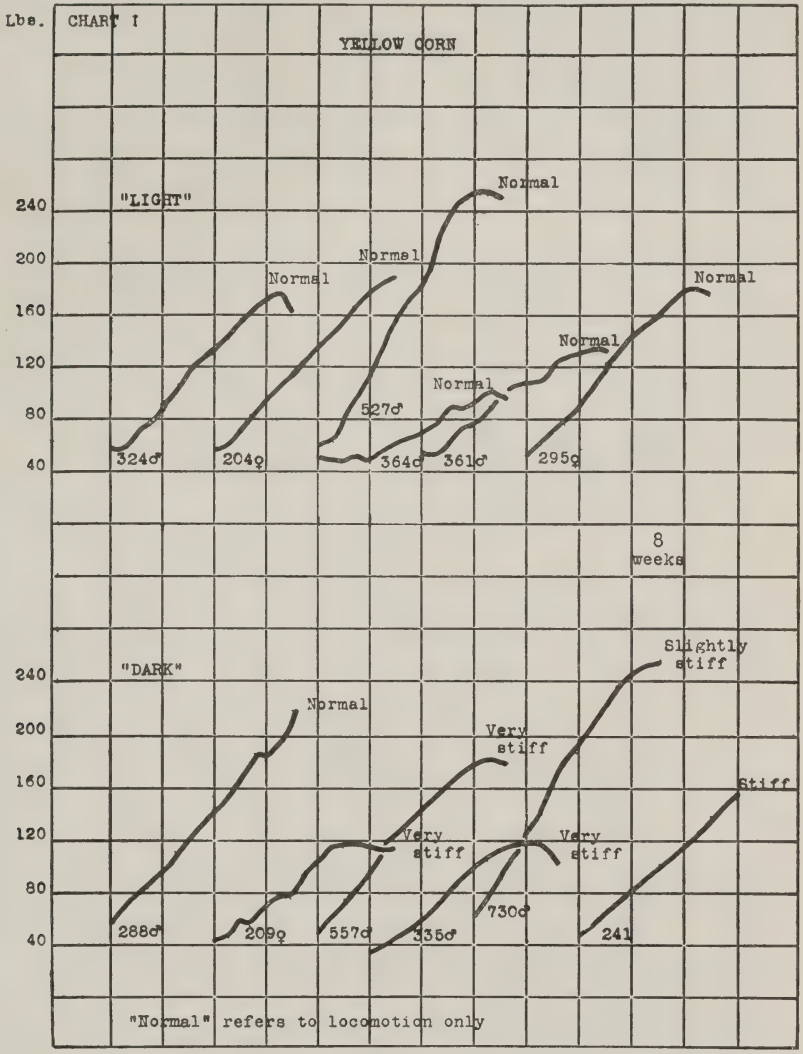

Chart I.

definite data on the age of the pigs, the number in each litter, the ability of the mother to suckle them, and the rations fed, it is

not surprising that growth on the experimental ration was so variable. It appears, furthermore, that in our animals the fac-

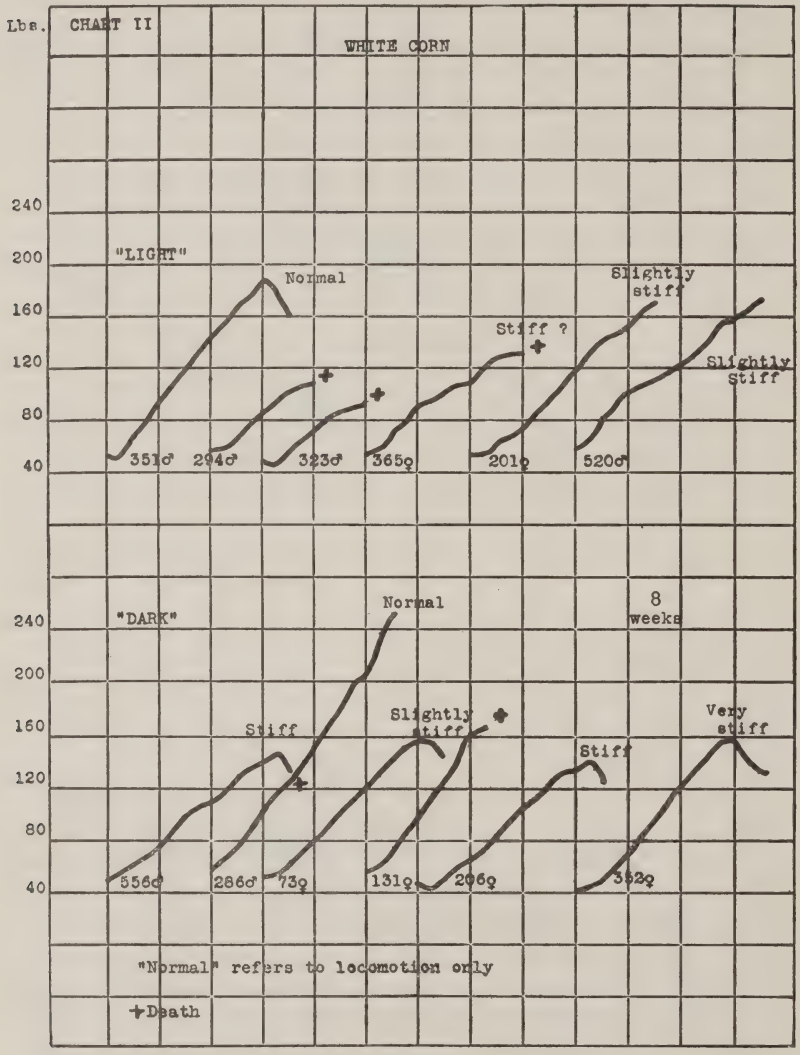

CHART II.

tor of infection even during the early growth period may not have been entirely excluded because five animals died from pneumonia

before the termination of the experimental period and at slaughter all but one of the animals had signs of chronic or acute pneumonic infections of varying severity. It so happens, however, that the exception (Pig 361) with normal lungs grew very poorly, so it becomes questionable how much of a rôle lung infections played in our growth retardations. It is, of course, well known that lack of vitamin A predisposes to pneumonia and in spite of the chronic nature of many of the cases as revealed by post-mortem it is doubtful if this condition was prevalent early in the experiment when growth was already inhibited.

In general, growth was better on yellow corn than on white corn, which bears out the original findings of Steenbock and Boutwell (14) on rats, and Morrison, Fargo, and Bohstedt (16), who with the authors' counsel carried out experiments with swine demonstrating the same point. That the growth difference was not pronounced, is to be associated,—with the irregularities, to a dominating effect of vitamin reserves; in fact, the very pronounced abnormal growth of some individuals can be correlated with variations in blood and bone analysis, as will be pointed out later—all indicating the effect of vitamin stores.

As to the effect of light on growth our experiments give us no information which is surprising in view of the findings of Hume (5), Goldblatt and Soames (17), and Steenbock and Nelson (6) which indicated such relations beyond question in the rat when according to the latter a deficiency of the antirachitic vitamin prevails. It might be possible that to a certain extent, in the white corn group, deficiency of vitamin A in the ration militated against such revelations, because obviously with a dominant deficiency of vitamin A growth would not be possible, but the facts of the case are, that growth was sufficiently long continued, with the amount of vitamin A present, for light to have exerted its complemental effect long before complete failure supervened. To a lesser extent, the same situation existed with respect to the yellow corn animals.

As the experiment progressed it became increasingly evident, that in the yellow corn group, light had a decidedly beneficial effect upon the animals because little by little, first temporarily and ultimately permanently, all but one of the animals in the dark developed the so called "rheumatism" marked by vary-

ing degrees of stiffness in either the front or hind quarters while those kept in the light were perfectly normal. It made it all but impossible for some of the animals to get up or walk even upon extreme provocation. The one exception, Pig 288, evidently had enough vitamin stored so as not to need the light, as borne out by data submitted later. When stiffness occurs in isolated instances in slight degree, not too much emphasis is to be put upon it because obviously lameness as a condition may have varied origin. In advanced cases, however, the condition becomes quite typical, but the observer is always decidedly handicapped—much more so than with most experimental animals—in comprehending what the pig may be experiencing subjectively. In no sense of the word, however, was paralysis ever observed.

On the white corn ration the evidence is confusing. Death from pneumonia was one disturbing feature which killed off three in the light and two in the dark. The pigs in the light were, on the whole, more exposed to cold because, while the barn was heated, the western exposure of their door, continually open, allowed the cold western winds to sweep into their pens. This, we have no doubt, is what caused more animals of the light group to succumb to pneumonia,—both groups, of course, being rendered susceptible to it by the low vitamin A content of the ration. The occurrence of stiffness was irregular because it appeared that Pigs 201 and 520 in the "light" group, as well as all but one, namely Pig 286, in the "dark" were afflicted. Pigs 351 and 286 were entirely normal.

Bone Analysis.

Years ago in the study of nutritional factors in relation to bone metabolism it was customary to arrive at conclusions from chemical analysis of bone, sometimes for ash only, but at other times more detailed analyses were made for calcium, phosphorus, magnesium, and sometimes for sulfur as well. Examination of the literature reveals, however, a surprising amount of variation in the percentage composition of the bones of different animals of the same age and size, and on the same diet which is not at all satisfactory from the standpoint of the analyst. This fact has led to search for other methods and has led to the adoption of the technique of the histologist for the determination as to

whether calcium has been normally deposited or not. Unfortunately bone pathology is a field with which the pathologist ordinarily is not often brought in contact, and the proper designation of the different processes taking place in normal as compared with pathological bone is not a matter of common knowledge. This has led to considerable confusion among investigators. The drawbacks of the general use of histological interpretation lie not only in the opportunity for wrong diagnosis but in the impossibility of quantitative expression of values. For this reason Bethke, Steenbock, and Nelson (18), and Steenbock, Jones, and Hart (19), turned again to chemical analysis of bone and with it have obtained excellent concordant results. It is our opinion that the difficulty experienced by others has been largely due to the fact that no consideration was given to the variations in lipoidal material in bone.

The bones used for our analysis were left humeri except in three cases where upon death of the animal the femurs had been removed instead. They were dissected out practically free from muscle and then dried at 86°C. for about a week. They were then crushed in a screw-press, wrapped in cloth, and extracted for approximately 48 hours in large Soxhlets with hot alcohol. After drying, they were weighed and incinerated in an electrically heated muffle furnace to a white ash.

The results of these analyses are shown in Table I. The variations are not great, but in general it is seen that sunlight promoted calcification in both groups. We have in these data only a very limited picture of the effect of light, because, as pointed out before, very little light was received by the "light" groups in the last few months of the experimental period. This with the maturing of the animals and the corresponding decrease in immediate calcium requirement worked to even out differences. It is interesting to note that Pigs 288 and 286 kept in the "dark" and both free from stiffness had the highest ash content which again testifies to their irregularity in the series.

Blood Analysis.

Blood samples for inorganic phosphorus were taken for the first time November 21, 5 months after the beginning of the experiment, at which time three of the pigs in the white corn groups had already died from pneumonia. The samples were taken by

tail bleeding, removed to the laboratory, and, after standing over night in a refrigerator for separation of serum, were analyzed for inorganic phosphorus by the Briggs-Bell-Doisy method (20). These analyses were run because Howland and Kramer (21), Hess and Unger (22), von Meysenbug (23), and György (24) showed that in rickets the inorganic phosphorus of the blood is reduced. On the basis of their evidence Bethke, Steenbock, and Nelson (18)

TABLE I.

Percentage of Ash in Bones.

Yellow corn.		White corn.	
Pig No.	Ash.	Pig No.	Ash.
	Light.		
	per cent		*per cent*
527	51.3	365	50.6*
204	53.0	520	52.1
324	53.3	201	52.2
361	53.9	351	57.6
364	54.3	294	No sample.
295	56.8	323	" "
	Dark.		
209	42.8	556	45.6*
355	46.2	206	48.2
557	48.2	352	50.8
730	51.2	131	51.0*
241	51.3	73	52.1
288	55.0	286	57.1

* Designates femurs which were removed upon death of the various animals and, therefore, represent less mature bone as seen by inspection of Chart II.

and Steenbock, Hart, Jones, and Black (25) have already used this method in studying various problems in connection with the antirachitic vitamin. On the same basis it appeared profitable to use this method in the diagnosis of the condition of the pigs looking especially for differences in phosphorus in the groups kept in the "light" as contrasted with the "dark."

The first results are shown in Table II which is self-explanatory. In all cases the animals on yellow corn showed a pronounced de-

crease in inorganic phosphorus with confinement in the "dark." On white corn the difference was not so great due mostly to the fact that with confinement in the "dark " the phosphorus was not depressed so much as it was on the yellow corn ration. This emphasizes what Bethke, Steenbock, and Nelson (18) touched upon in a previous communication, that the depression in inorganic phosphorus cannot be used as a simple infallible index of the existence or of the severity of a case of rickets. Undoubtedly

TABLE II.

Inorganic Phosphorus in Blood Serum—November 21, 1923.

Yellow corn.		White corn.	
Pig No.	Inorganic P per 100 cc. serum.	Pig No.	Inorganic P per 100 cc. serum.
	Light.		
	mg.		*mg.*
324	4.70	520	5.06
204	4.72	351	5.11
295	4.98	201	5.44
527	5.62	365	6.54
361	5.88	323	Dead.
364	6.24	294	"
	Dark.		
209	2.46	206	3.20
355	2.55	556	3.52
557	2.77	73	4.35
730	3.39	286	5.88
241	3.94	131	Dead.
288	No sample.	352	No sample.

it is true that sooner or later a decrease in inorganic phosphorus occurs, as the disease runs its course, but in the absence of information that other diseases may not also bring about this depression its value for diagnosis must be questioned.

In fact Gerstenberger, Burhans, Smith, and Wetzel (26) have already reported a decrease in inorganic phosphorus during pneumonia when cod liver oil was being given. From this standpoint the values for inorganic phosphorus obtained at the time of termination of the experiment and shown in Table III are not at all

consistent, because Pig 361, the only pig with normal lungs, gave the lowest value among the animals in the "light" on yellow corn. For the present then one must be content with pointing to the general relations which, though not so conclusively as before, show that the inorganic phosphorus of the animals kept in the light was higher than of those in the dark. In the case of unusually high values obtained from those kept in the "dark," *e.g.* Pigs 288 and 286, it is interesting to note that these were not stiff when the samples were taken.

TABLE III.

Inorganic Phosphorus in Blood Serum—January 10, 1924.

Yellow corn.		White corn.	
Pig No.	Inorganic P per 100 cc. serum.	Pig No.	Inorganic P per 100 cc. serum.
	Light.		
	mg.		*mg.*
361	3.60	520	3.30
364	3.74	351	3.44
527	3.98	201	5.50
324	4.50	323	Dead.
295	4.97	294	"
204	6.05	365	"
	Dark.		
209	2.60	206	2.66
241	2.99	352	2.71
730	3.11	73	3.69
557	3.21	286	6.37
355	4.01	556	Dead.
288	5.40	131	"

Histological Examination.

With the termination of the experiment, costochondral junctions were taken from all the animals for histological examination. These were sectioned and stained with hematoxylin and eosin after decalcification.

The bone pictures revealed were varied in character especially in the case of the animals on white corn irrespective of the light treatment. This is probably what was to be expected due to the

multiple deficiency of the ration and the resultant pathological condition of the animals. There was, however, considerable uniformity in the yellow corn groups. The sections showed a regularity of structure and arrangement in the group exposed to "light" which was not found in those kept in the "dark." In the first place, the line of demarcation of osseous tissue from cartilaginous tissue was smooth and regular in all cases except in Pig 364 where some irregularity was shown at the sides. Of those kept in the "dark" this line was jagged, marked by a persistence of tongues and islands of cartilage in the primary spongiosa. With this there obtained great irregularity in the columnar arrangement of the cartilage by way of contrast with sections taken from animals kept in the "light" where the orientation was uniformly orderly. Hypertrophy of the cartilage cells was shown by the animals in the "light" in a narrow band, varying from a minimum of from 3 to 5 cells to a maximum of 6 to 7 cells, again with the exception of Pig 364 where on one side of the junction examined there existed a zone of hypertrophic cells 30 to 40 cells deep. With the animals kept in the dark the values ranged from a minimum of 3 to 7 to a maximum of 6 to 15 with probably an average of 10 for the different sections. There was also a marked difference in the nature of the spongiosæ. In the animals kept in the "light" the differentiation between primary and secondary spongiosa was distinct, while in the animals kept in the dark the primary spongiosa was irregular and often coarse with a fusion of the two zones.

At the present stage of investigation little is to be expected of the histological examination partly because our animals were killed when too old and partly because too little is known of the specific nutrient requirements of this species. Golding, Zilva, Drummond, and Coward (12) have discussed whether or not in their animals rickets actually was present. They were doubtful of its occurrence because of the absence of a histological picture characteristic of this disease. In a way such a discussion appears in our work to be beside the point not only because we now know that growth is necessary for the production of this picture, but also because we obtained such pronounced improvement with light. We prefer to take the attitude that rickets is a pathological entity distinct from the abnormal relations observed in the mature

animal (27) *only in that growth brings about the production of a special histological picture.* Qualitatively the needs of the growing animal and the non-growing, whether adult or immature, are the same; they both need a suitable balance of calcium and phosphorus, and the antirachitic factor. This point of view is bound to simplify experimental investigation and cannot, to say the least, be any farther from the truth than the present archaic practice of completely isolating rachitis with respect to its causal relations. Such a point of view is most certainly bound to attract more general attention to the study of calcium, phosphorus, and the antirachitic factor in their relations to the requirements of the animal.

DISCUSSION.

It appears from our data that light, in the absence of a sufficiency of the antirachitic vitamin, is an important factor to consider in swine husbandry. In fact there remains no question, in view of the conditions under which pigs are generally kept and fed in northern climes, that more attention should be paid to illumination. This point must be emphasized, because, while we have here given consideration to light, only in its relation to the welfare of the animal during its actively growing period, it is probable that it is of importance in connection with gestation and lactation as well. This statement is made because it is coming to be appreciated more and more that failure of the animal to function normally in these capacities can often be related to interference with proper assimilation and storage of lime. Rickets as a phenomenon of deficient calcification of bones can be looked upon as a disturbance in the growing animal of the physical-chemical equilibrium between phosphoric and carbonic acids as related to calcium, which leads to interference with the deposition of the calcium phosphate-carbonate complex. It is not unexpected from this point of view that influences which tend to affect this equilibrium should have their effect on the production of the disease. In harmony with this Sherman and Pappenheimer (28) have reported the prevention of rickets,—induced in rats by feeding the Sherman and Pappenheimer diet, by the administration of dipotassium phosphate. They emphasize the fact that this does not imply "that in these cases the cause of rickets was necessarily

a deficiency either of potassium or of phosphorus. The quantitative relations of the inorganic ions rather than an absolute deficiency of any one of them, may have been the determining factor." Zucker, Johnson, and Barnett (29) state that a decrease in the acid-base ratio may lead to the development of rickets-like changes in the bones of rats. McCollum and coworkers (30) state that the absolute amount of calcium and phosphorus and also their proportion exerts an effect on calcium deposition in bone.

It has been shown by Bethke, Steenbock, and Nelson (18) that when growth is retarded by the absence of a sufficiency of the antirachitic vitamin the addition of an excess of lime may correct the difficulty. They have pointed out that many of the beneficial results of calcium administration reported in the literature may have had their origin in this reciprocal relation which, within certain limits, exists between calcium and the vitamin. It is entirely probable that the corrective action of legume hay and meat scraps observed by Hart and coworkers (9) may have been due to calcium as well as to the antirachitic vitamin.

In practice the specialist in feeds and feeding will desire to have available a ration compounded from naturally occurring foodstuffs which can meet the requirements of the animal irrespective of the action of light. Unfortunately the distribution of the antirachitic vitamin is not as yet well worked out, but from the practical experience of hog men as well as from unpublished work of this laboratory on rats and chickens one is led to believe that grains generally do not contain much of it. Fresh green plant materials, on the other hand, as shown by the work on goats carried out in this laboratory, are rich in it (27). Even green roughages such as clover or alfalfa, carefully cured, contain considerable amounts of this vitamin so that it is not at all improbable that with such materials the pig's requirements can be satisfactorily met. These roughages are already being extensively used as a supplement for vitamin A, so that it merely remains to determine the amounts which can be satisfactorily taken care of without taxing the animal's capacity to handle such bulky materials. Milk as a supplement is probably not satisfactory as neither butter fat nor skimmed milk contains much antirachitic vitamin. In English laboratories cod liver oil has been used to feed pigs,

cows, and calves with good results (31). It is questionable, however, if this oil will ever find a general application for this purpose in the United States though it is not excluded that with the development of a demand for oils possessing this property, other oils just as active will be conserved for this purpose as by-products from the fishing industries.

If light is to be depended upon entirely or in part as the antirachitic agent the question arises as to what degree of illumination and what kind of illumination can be depended upon as being effective. Here again we can draw upon the experience of the clinician and the experimentalist working with laboratory animals. Hess and Weinstock (32) in experiments with rats, using the quartz mercury vapor lamp and various light filters came to the conclusion that light to be an effective agent must have a wave-length not longer than 3030 or 3120 Å.u. This excludes sunlight transmitted through glass, for while solar radiation reaching the earth contains ultra-violet rays as short as 2900 Å.u. ordinary window glass filters out all rays shorter than 3300 Å.u. so that no effective rays remain. Hess (33) has, as a matter of fact, confined rats in an enclosure of ordinary window glass and thus exposed them to the rays of the sun; he observed no protective action. It will be noted in our experiments that all our pigs were kept in the diffuse light of the barn. This means that the animals must be exposed to sunlight directly if use is to be made of solar radiations for antirachitic purposes. As an alternative there remains the use of the direct rays of artificial light, both the quartz mercury vapor lamp and the open carbon arc having been found effective.

CONCLUSION.

Gross symptoms supported by chemical analysis of blood and bone as well as by histological evidence indicate that sunlight is a factor of economic importance in the production of pork under confined conditions in northern latitudes.

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CHANGES IN THE HYDROGEN ION CONCENTRATION OF THE BLOOD WITH COAGULATION.*

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Studies inquiring into the mechanism of blood clotting have been made by many careful investigators, and while much is written regarding the factors concerned, there is offered as yet no satisfactory and uniformly accepted explanation for the fact that blood usually clots when it escapes from a wound. Some of this confusion exists, perhaps, because the more recent investigations in colloidal chemistry were not available, and because some of the factors concerned with gel formation have not been understood. These last mentioned observations, especially, are significant since with them consideration has been taken of the importance of the H ion concentration of the gel medium, a factor whose relation to blood clotting has not been apparent and which to date seems to have received little attention. The work of Loeb (1) and others regarding the importance of the H ion concentration of the medium upon the behavior (ionization) of the proteins in solution and of the electrolytes as well, brings into studies of blood clotting this factor for consideration.

Much of the work on blood clotting concerns the isolation of certain substances and the evaluation of their importance in the clotting mechanism. According to Bordet (2) coagulation of the blood is the aggregation into meshes of fibrin of particles of fibrinogen which Fredericq, 46 years ago, found in the plasma dispersed as a colloid. This substance, whether so named or otherwise, is accepted quite generally as the coagulating part of the blood clot. Another important constituent of the fibrin clot is calcium. Many years ago, Brücke (3) demonstrated that fibrin left an ash containing calcium phosphate. Pekelharing and Hammarsten (4)

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found that these salts are not necessary for the transformation of fibrinogen into fibrin by the thrombin, but that they are indispensable to the formation of the latter from a precursor in the blood. Bordet and Gengou (5) long ago showed that contact accomplishes in result the effect of calcium ions; *i.e.*, that contact with a foreign solid body brings about the appearance of thrombin, but is not requisite for the coagulating influence of the latter. When blood is received into a paraffined vessel no thrombin is formed; when received into an unprotected vessel thrombin is produced at the zone of contact, a fact which explains why coagulation begins along the wall. Blood platelets are believed to furnish a lipoid substance (cytozyme) which, mixed with a substance in the serum (serozyme), gives rise in the presence of calcium ions to thrombin, the substance that gels the fibrinogen. According to Howell (6), the circulating blood contains an antithrombin which, in shed blood, is neutralized before coagulation takes place.

This brief statement regarding the theories of blood clotting makes apparent that little consideration has been given to changes in H ion concentration which follow when blood is removed from its normal position in the blood vessels and comes into new relations as regards gas equilibria (carbon dioxide especially). In the adjustment to these new relations there may be changes in base (OH) and acid (H) distribution which are fundamentally important in blood clotting.

According to present ideas (7) regarding the formation of gels,—and the clotting of plasma belongs with these reactions,—the uniform precipitation throughout the liquid of a network which takes up the solvent strongly, is necessary. The resulting network of myriads of hydrous particles constitutes the gel structure. The amount of substance dispersed in the solvent and necessary to form a firm jelly is determined by the size of these particles and the extent to which they are able to occlude the solvent. Since the presence of finely divided particles with marked hygroscopic properties is necessary for the formation of a jelly, the precipitation of hydrous substances from their colloidal solution is most favorable for the formation of a gel. This occurs, however, only when the precipitation takes place at a suitable rate in a medium without appreciable solvent action, or where the substance is

being precipitated in a less soluble or insoluble form. The effect of salts on a gel formation is determined by the precipitating or stabilizing influences of their ions, in as far as these influence the amount and velocity of the precipitation. A slow precipitation is better if there be no increase in the size of the particles as the result of the solvent action by the electrolyte. Rapid coagulation by the addition of an electrolyte to a colloidal hydrous substance usually results in a gelatinous precipitate and not a jelly because there is no uniform mixing of the colloid with the coagulant. The slow, uniform precipitation necessary for gel formation is replaced here by a rapid, uneven coagulation, and the consequent contraction which distinguishes a gelatinous precipitate from a jelly. When gel formation is prevented by the presence of a stabilizing ion, the addition of an electrolyte having a suitable precipitating ion neutralizes the stabilizing ion and a gel forms. This result obtains also when gels are formed by dialysis. Here the stabilizing ion is removed slowly and uniformly to a concentration below that necessary to hold the colloid in solution.

Weiser and Bloxsom (7), in their studies of arsenate jellies prepared with aluminum, iron, and manganese salts, observed gel formation upon the neutralization of a stabilizing H ion by either suitable acids,[1] alkalies, or salts added in definite concentrations. If too little of the electrolyte was added no jelly or an imperfect jelly formed, while, if too much was used, a gelatinous precipitate was obtained. Their results with manganese arsenate jellies are instructive. Potassium dihydrogen arsenate ionizes as follows:

$$KH_2AsO_4 \rightleftharpoons K^+ + H_2\,AsO_4^-$$

On account of the solubility of $Mn(H_2AsO_4)$, no Mn ions are permanently removed from solution by the interaction of $MnSO_4$ with H_2AsO_4. However, the latter ionizes secondarily to a slight degree as follows:

$$H_2AsO_4' \rightleftharpoons H' + HAsO_4''$$

Insoluble $MnHAsO_4$ is formed, with sulfuric acid as a cleavage product. Since the precipitation of $MnHAsO_4$ is accompanied by

[1] "The H-ion concentration in the solution might even be increased by the addition of an acid with a multivalent anion that is strongly adsorbed provided the solvent action of the acid is not too great."

the formation of an equivalent amount of H ions in solution, an equilibrium is set up which prevents the complete precipitation of the manganese. Using the primary (KH_2AsO_4) salt with the manganese sulfate, complete mixing was obtained regularly before precipitation started, but with the secondary and tertiary salts a gelatinous precipitate or cloudy, heterogeneous jelly was produced instantly. This emphasizes again the need of slow neutralization of the stabilizing ion for gel formation in a medium. Kraemer (8) observed, in other arsenate jelly studies, optimum ratios between the interacting manganese sulfate and the potassium dihydrogen arsenate. The sulfuric acid formed in this reaction Kraemer regards as increasing and favoring the formation of the jelly.

Other gels are commonly prepared from fruit juices. Tarr (9), in determining the acidity factor in fruit juices, finds that there is a direct relation between jelly formation (gelling of pectin) and the actual acidity or hydrogen ion concentration. The minimum H ion concentration at which jelly forms is pH 3.46 for the purest obtained pectin, and the H ion controls the formation of the jelly. When the minimum H ion concentration has been reached, the jelly forms, and as the reaction becomes more acid (up to pH 3.1), the jelly becomes stiffer. At a H ion concentration greater than pH 3.1 contraction of the gel occurs.

Similar notions regarding the transformation of the fibrin from a hydrosol to a hydrogel state have been expressed in a number of recent reports. Thus Hekma (10) subscribes to the physical theory of blood coagulation and thinks the gelling process is due to the formation of acid compounds such as nucleoproteins, globulins, and phosphatides from the platelets and leucocytes as they separate from the plasma. These withdraw alkali and water from the plasma until the fibrin is changed from a sol to a gel. With the ultramicroscope this process appears as the formation of minute particles which grow into threads. Kugelmass (11), starting with a plasma-thrombin or a fibrinogen-thrombin system of pH less than 7.0, observed a diminution in the H ion concentration which resembles an adsorption process, being very rapid at first. The fibrin clot, he says, has a lower H ion concentration than that of the original mixture. The greater the original H ion concentration the larger is the differ-

ence between the initial and final pH. On the average, about 50 per cent of the H ions disappear during the coagulation. The optimum for coagulation is between pH 5.0 and 8.0. On both the acid and the alkaline sides of the optimum range, the greater the deviation the less perfect is the coagulum and the fibrin remains in suspended shreds. In another report on changes in the H ion concentration during coagulation of the blood, Kugelmass (12) says that when the clot forms it adsorbs free ions, and that the H ion concentration of the medium in which clotting occurs is always less than the original mixture.

The results by Kugelmass on changes in the H ion concentration of the blood during coagulation, while important, were not obtained with blood in the usual process of coagulation. In systems where the normal carbon dioxide content of the blood is disregarded, significant and rapid changes in reaction may be overlooked. A method for demonstrating H ion changes in rapidly clotting blood needs as essentials, speed and the detection of minute variations. The difficulty encountered in making such determinations by the gas chain method is chiefly the time interval needed for the system to come into equilibrium. During this period coagulation takes place and the platinized electrode becomes gummed. It seemed possible, then, to obtain the speed and accuracy by means of a colorimetric system carefully standardized and suitable for detecting minute changes in reaction. Recently, Brode (13) and Holmes (14) have described a method for determining the hydrogen ion concentration of a medium by the spectrophotometer. According to their work, especially that of Brode, changes within the pH range of a 2-color indicator, as for example one with green-yellow in the acid range and red in the alkaline, are accompanied by changes in the amount of each color measured at its characteristic wave-length, rather than in a sequence of colors in transition with each other over the entire pH range. Thus with a certain indicator, a particular and characteristic wave-length is used on the spectrophotometer, and with a definite thickness of the analyzing cell in the presence of a determined concentration of the dye, the amount of color transmission is measured. Standardized by buffer solutions carefully calibrated electrometrically, the amount of color expressed as percentage or in terms of extinction coefficient may be converted into an

equivalent pH value. A Keuffel and Esser[2] color analyzer was used for making the colorimetric determinations (made in measurements of color percentage), and cresol red, having a pH range of 6.4 to 9.4, was chosen because the pH values of the blood are approximately in the middle portion of the indicator range. In chosing an indicator for a certain pH range this feature ought to be considered, for, in the middle portion of the range, the color percentages and the pH values are practically linear and parallel. The amount of indicator contained is also important in order that the readings in percentage (with the spectrophotometer used) come within the range of 0 to 100. If the amount of dye used is too small the pH curve will be distributed over a range more than 0 to 100 per cent, and if too much, the percentages of color will read too low. The amount of color is dependent upon the thickness of the fluid in the analyzer cell, generally speaking, according to Beer's law.

All of the results obtained in this study are from measurements made in 1 cm. length cells, the amount of cresol red being 0.02 mg. in 1.9 cc. volume. The standardization of these measurements was accomplished with buffer solutions whose pH values were ascertained electrometrically by the gas chain method with a Leeds and Northrup, Type K potentiometer. The results of such standardization measurements in terms of color percentage measured with the color analyzer at wave-length 572 $m\mu$ are given in Table I. These results suggest that within the pH range 7.68 to 7.25 the pH variations and the color percentage variations are practically equal and uniform and that from these values the intermediate ones may be interpolated to make a scale. So constructed the values form Table II. In making Table II, as was mentioned, the middle portion of the indicator curve is almost linear, but at each end this relation does not hold, and where percentage values are obtained in these portions corrections are necessary. However, in the work reported here most of the values obtained are in the middle portion of the indicator curve.

The blood of rabbits was taken from the heart under oil through a paraffined needle into a paraffined syringe, transferred quickly under oil to a paraffined centrifuge tube, and centrifugalized. The

[2] This instrument was purchased with a sum of money given by the Winfield Peck Memorial Fund.

unclotted plasma was removed and without delay mixed with the indicator in a closed color analyzer cell. The escape of carbon dioxide was prevented as much as possible by transferring the serum under oil in a paraffined pipette. The cell was then closed quickly, and readings were made at once. After clotting under

TABLE I.

Electrometric pH.	pH difference.	Color analyzer.	Difference.
		per cent	*per cent*
7.68		30	
7.57	0.11	39	9
7.40	0.17	56	17
7.37	0.03	59	3
7.33	0.04	62	3
7.25	0.08	68	6

TABLE II.

pH	Color.	pH	Color.	pH	Color.	pH	Color.
	per cent		*per cent*		*per cent*		*per cent*
7.70		7.60	36	7.50	46	7.40	56
7.69		7.59	37	7.49	47	7.39	57
7.68	28	7.58	38	7.48	48	7.38	58
7.67	29	7.57	39	7.47	49	7.37	59
7.66	30	7.56	40	7.46	50	7.36	60
7.65	31	7.55	41	7.45	51	7.35	61
7.64	32	7.54	42	7.44	52	7.34	62
7.63	33	7.53	43	7.43	53	7.33	63
7.62	34	7.52	44	7.42	54	7.32	64
7.61	35	7.51	45	7.41	55	7.31	65
7.30	66						
7.29	67						
7.28	68	Cresol red, 1 cm. length tube.					
7.27	69						
7.26	70	0.02 mg. in 1.9 cc. volume.					

oil, another reading was made with fluid expressed from the clot. With some, the plasma was removed from the sedimented cells and placed in another clean tube to clot under oil, with others the clotting occurred in the presence of sedimented cells. The results seem to be alike. They are collected in Table III.

These results, obtained according to the methods given, demonstrate that blood clotting under the conditions mentioned is accompanied by a slight diminution in alkalinity. The contention may be made that plasma changes in reaction in a like manner simply upon standing. This, however, has not been observed in these experiments, for with some plasmas where clotting did not take place for 30 minutes the pH values obtained were the same at the beginning and at the end of that period, while readings of unclotted and clotted plasma made during like intervals of time had the pH differences mentioned. Serum obtained from clotted plasma and measured at intervals for 7 hours remained unchanged.

TABLE III.

pH before clotting.	pH after clotting.	pH difference.
7.46	7.36	0.10
7.54	7.43	0.11
7.46	7.36	0.10
7.39	7.35	0.04†
7.41	7.32	0.09†
7.40	7.35	0.05†
7.51	7.36	0.15
7.38	7.26	0.12
7.41	7.29	0.12
7.56	7.46	0.10
Average.....		0.09
7.58*	7.34	0.24

*Dog serum.
†These plasmas clotted poorly.

DISCUSSION.

The results detailed here may have significance in correlating the clotting of blood with other gelling processes. In the formation of these other gels, acids in proper concentrations are able to cause the gelling process to take place. With some gels, of course, the cleavage of an acid radical from the hydrosol is part of the process. This possibility has been considered in the clotting of blood. Ruppel (15) has suggested that the euglobulins, to which class the fibrinogen belongs, are split from an acid constituent of the serum at their isoelectric point, and Kugelmass (11) has de-

termined the isoelectric point of fibrin to be at pH 7.2. The fact remains, however, that acids in proper concentration do bring about the gelling process and, in terms of gel formation, are regarded as neutralizing the stabilizing ion of the hydrosol, thus permitting the hydrogel to form. The experiments by Kugelmass show that H ions are used up or bound in the gelling process. As regards the source of this acidity, taking into account that it forms with great rapidity, carbonic acid as a possible source deserves consideration. Bordet, in his review, has emphasized the importance of contact with rough surfaces or particles in blood clotting, and that the clotting of plasma begins in those portions touching these surfaces. Similar reactions are commonly observed in liquids supersaturated with a crystalloid, when simply scratching the side of the containing vessel or introducing a crystal or particle sets into action the whole crystallization process. The separation of fibrin, which is formed from its precursor, fibrinogen, probably should be considered as the precipitation of a relatively insoluble compound. When the blood is brought into conditions of atmospheric tension, as regards carbon dioxide there is a supersaturation. Although bound in the blood largely as $BHCO_3$[3] a rearrangement takes place under the conditions of atmospheric tension and CO_2 escapes. This reaction may be represented in the following way.

$$2BHCO_3 \rightleftharpoons B_2CO_3 + H_2CO_3$$

$$H_2CO_3 \rightleftharpoons H_2O + CO_2$$

The instability of the carbonic acid, however, permits only a transient effect, but since the reaction of serum exposed so as to allow carbon dioxide to escape becomes considerably more alkaline (0.5 pH or more), appreciable H_2CO_3 is liberated under the altered conditions. This liberation ocurrs without base to hold it neutralized during the process. In other words unbound, transiently present acid of considerable strength (H ion) is formed. Conditions of this sort, of course, are favorable for gel formation in the presence of a dispersed hydrosol, as has been observed in the experiments mentioned with other gelling processes.

[3] The letter B indicates in the formulas a single valence of a base.

The intravascular clotting of blood post mortem may follow along similar lines and may resemble the rigor appearing in muscles after death. It is known generally, and has been for some time, that in striated muscle tissue a postmortem rigor is accompanied by the appearance of an acid, by some regarded as lactic acid. Studies of rigor mortis in smooth muscle tissue (fibromyomas) by Hirsch (16) revealed that in those tissues not manifesting such a change in reaction no rigor appeared. Since acid substances appear in tissues after death, it is possible that these become important in causing the intravascular blood clotting post mortem.

Perhaps of some significance are those observations on delayed blood clotting in animals during peptone and anaphylactic shock, as well as after intravenous injections of foreign proteins or bacteria. With all of these a variation of the hydrogen ion concentration of the blood is reported. With peptone shock Menten (17) observed a marked increase in the acidity of the blood and blood plasma. Under conditions such as these the alkaline reserve is markedly diminished, and the acidosis, so called, is compensated, the concentration of free H ions being no greater. When such blood is exposed to air the amount of $BHCO_3$ is relatively small, as is also the amount of H_2CO_3 liberated under the diminished carbon dioxide tension. This difference in the amount of available H ions may be important in the slow clotting or the incoagulability of such bloods.

SUMMARY.

The clotting of blood plasma under conditions where the escape of carbon dioxide is largely prevented is accompanied by a sudden slight diminution of its alkalinity.

With rabbit plasma an average pH difference of 0.09 was determined between clotted and unclotted plasma.

Those plasmas with only slight changes in pH clotted poorly.

The possibility of sudden changes in carbon dioxide tension initiating the normal clotting of blood is considered.

Dr. E. R. LeCount suggested to me the possibility of there being pH changes with the clotting of blood. We have discussed the results obtained as the work progressed. His helpful suggestions are hereby acknowledged.

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STUDIES OF AUTOLYSIS.

XII. EXPERIMENTAL ATROPHY OF MUSCLE TISSUE.

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Muscle atrophies are known of both physiological and of pathological types. As examples of normal or physiological muscle atrophies we have the changes in the uterine wall after parturition, the skeletal muscle atrophies of disuse, of starvation, and of old age. The complete atrophy of the tadpole's tail at metamorphosis and similar atrophies in related lower animals, are examples of complete normal atrophies. A striking example of an atrophy furnishing fuel and material for large development of other tissue is seen in the muscle atrophy of the migrating salmon as the spawning period is reached.

Examples of pathological atrophies follow lesions of the central nervous system such as anterior poliomyelitis, myelomalacia, or of the degeneration of peripheral nerves following injury. Ankylosis of the joints and fixation by splints produce atrophies that are in a sense physiological; that is, they are atrophies of disuse.

Cardiac atrophy is believed to be always pathological though the gradual shrinkage of the heart in old age may be called physiological.

Occlusion of primary arteries to a muscle may produce death and necrosis, as in the cases of Volkmann's "ischemia," while occlusion of the vein is regularly followed by loss of function, degeneration, inflammation, and fibrosis—as was shown by Brooks (1). In his studies on the relation of circulatory changes to pathological changes in muscle Brooks noted a number of instances where a muscle, with sufficient reduction of its blood and lymph flow, was completely resorbed in 7 to 12 days. Such

a rapid and complete removal of muscular tissue is in striking contrast to the small autolysis of the normal tissue as described in a previous paper (2), and we must assume an additional proteolytic mechanism in such a case. What part the leucocytes played in this type of removal is not clear from the experiments reported, though apparently there was no evidence of an acute inflammatory process. These results suggest interesting further experimentation to develop the mechanism involved.

Muscular atrophy may be induced experimentally in mammals by section of the spinal cord (3, 4), or removal of the cerebral cortex (5), or most easily by section of a peripheral nerve supplying a group of muscles. Tendotomy will also lead to muscular atrophies (6) almost as extensive as those produced by denervation.

The results of such investigations have been largely of histological character. Connective tissue is found to increase, appearing more vascular and with more nuclei than in the normal. Adipose tissue also increases. The nuclei of muscle fibers themselves undergo marked proliferation (4, 7), so that the sarcolemma tube may appear filled with nuclei in the late stages (8). As a rule the fibers diminish in size, with an occasional hypertrophied fiber among them (9, 10). There is a difference of opinion in regard to the persistence of the striæ in voluntary muscle. Stier, Strümpell, and Jamin found them to persist, while others describe degenerating changes (7, 9, 11–13). Durante believed a "plasma regression" took place with hyperplasia of the undifferentiated protoplasm, longitudinal division of this into thin fibers, and "metamorphosis" into adipose and interstitial connective tissue.

A number of chemical changes have been reported in muscle atrophy. For example, Steyrer described alteration in the proportion of myosin to myogen. In the normal muscle the ratio is about 1:4, in atrophy 1:1.5. This increase in the myosin ratio begins on the 4th day following denervation and progresses (14).

Grund (15) found that the ratio of residual N to total N decreased, while the ratio of protein P to total P increased largely. This increase would appear to confirm the increase of nuclear substance relative to total mass of tissue.

Cathcart, Henderson, and Paton (16) found no significant change in creatine until after the 15th day, when a steady de-

crease of this constituent was observed. This seems to connect the creatine with the contractile substance of the sarcostyles rather than the sarcoplasm, since the substance of the sarcostyles is believed to diminish relatively early in muscle atrophy.

Rumpf and Schumm found fat increased to fifteen times the normal in a case of human polyneuritis (17). Grund (15) also found the ether-soluble fraction to rise from 6 to 17 per cent in experimental muscular atrophy in dogs. This means a 300 per cent increase in fat, while the total loss in weight is seldom as much as 50 per cent. This seems to establish the fact that there is an actual increase of fat.

Glycogen has been shown by Chandelon (18) to increase in atrophic rabbit muscles, and this was attributed to the loss of contractile function so that there was no longer the usual combustion of the carbohydrates. This fact has been confirmed by Vay (19). Audova (20) showed a slight but constant increase of water, Rumpf and Schumm (17) reported an increase of Na and Ca, but a decrease of K. These results alone do not give us any clear information about the general character of the changes which atrophy involves.

EXPERIMENTAL.

In the following experimental studies of atrophy we have attempted to develop the progressive changes in the muscle tissue with a view to correlating chemical and histological findings with the results of autolytic studies previously reported.

Healthy rabbits were operated upon under ether anesthesia and with aseptic technique. An incision was made along the line from the first to the third trochanter of the femur, about 2 to 2.5 cm. in length. The sciatic nerve was exposed, slightly beyond the piriformis muscle, and a length of 1 to 1.5 cm. removed. The femoral nerve was cut at the ligament before division and 1 cm. of it removed. The wound usually healed rapidly and only these animals were kept for purposes of the experiment.

It was found that a certain number of rabbits developed gangrene at the ankle joint or above, and these lesions frequently became putrefactive. A flannel-lined rubber shoe was used as a protection for the operated foot and prevented the incidence

of putrefactive changes. Injections of 95 per cent alcohol failed to prevent the development of gangrene. The rabbits were kept under good hygienic conditions and on a satisfactory diet.

At the end of a given period, the test animals were killed by injection of air into the ear vein. The large arteries were clamped and the muscles dissected as free as possible from connective tissue, blood vessels, and nerves. Tendons were cut at the point where muscle fibers were visible. A definite routine of technique in dissection was used in order to reduce as far as possible errors due to gross masses of connective tissue. Errors due to gangrene, fibrosis, adiposity, congestion, and edema cannot be eliminated from our data, but were checked in gross at dissection and later by histological study. In addition there are errors due to age, growth, sex, activity, general condition, etc., which cannot be controlled, but which may be reduced in effect by a sufficient number of animals. Before making the experiments reported here a large number of normal rabbits were killed and dissected, in order to establish our normal averages of muscle mass, and the expected individual variations. These preliminary dissections of normal animals made it possible to perfect the technique before any measurements of atrophic muscles were attempted.

Normal Muscles.

Two groups of muscles were removed, innervated by the femoral and the sciatic nerves. Right and left sides were dissected, following a standard technique, weighed, and compared. The muscle group innervated by the femoral nerve comprises the quadriceps femoris and sartorius. The group innervated by the sciatic contained the biceps femoris, semimembranosus, semitendinosus, and muscles of the leg and foot.

Table I shows that the maximum difference between the two sides is 2.4 per cent. In similar studies Langley isolated individual muscles rather than groups of muscles and found that 75 per cent of his 153 single muscles from seventeen rabbits, had a difference greater than 4 per cent, and only 9 per cent of them less than 6 per cent.

Audova isolated gastrocnemius, plantaris, and soleus and found the differences lay between 0.9 and 0.5 per cent. The dissec-

tion and comparison of a large group of muscles we believe more accurate than the dissection of the individuals of that group. Where we find, therefore, divergence between the operated and the normal control side of the animal, in excess of 2.4 per cent, we interpret it as being significant in our experiment.

Thirty-seven rabbits were operated upon as described. Four of these died from infection or failure of the wound to heal. Two died from pneumonia and a third from unknown causes. Thirty animals, therefore, survived the operation and were used for the studies of atrophy. These were divided into ten groups and killed at selected intervals. Sixteen of the thirty operated

TABLE I.

Comparison of Right and Left Muscle Groups of Normal Rabbits.

Body weight.	Weight of muscles on right side.	Weight of muscles on left side.	Difference from left.
gm.	*gm.*	*gm.*	*per cent*
1,010	27.4275	26.9025	+1.914
2,300	121.9040	119.0840	+2.319
1,770	59.6100	61.0225	−2.369
1,320	58.9300	59.7415	−1.377
1,490	44.0950	43.1500	+2.143
1,955	54.1435	54.4010	−0.476
985	47.5695	48.3820	−1.709
2,000	75.6045	76.8745	−1.680
1,280	38.8570	39.2255	−0.948
2,560	59.1755	58.8355	+0.588
Average difference.....			−1.5

rabbits developed gangrene—a traumatic lesion at the ankle joint. Four of these became putrefactive. Thirteen out of thirty developed swollen lymph glands in the operated leg, and seven of these had gangrene also. There seems, therefore, little relation between the incidence of gangrene and of swollen lymph glands. Twenty-one out of thirty rabbits showed congestion or venous hyperemia on the operated side. Only nine of the group are without apparent pathological changes, including congestion and edema in this term. They are fairly well distributed, however, three in 1 week, one in 2 weeks, two in 6 weeks, two in 7 weeks, and one in the 9 week period.

TABLE II.

Loss of Weight of the Denervated Leg of Rabbits.

Rabbit No.	Duration of experiment.	Initial body weight.	Weight when killed.	Muscles of legs.			
				Non-operated weight.	Operated weight.	Difference from non-operated.	Average difference.
	wks.	*kg.*	*kg.*	*gm.*	*gm.*	*per cent*	*per cent*
Control.	0			145.460	146.648	0.81	0.81
36	1	3.30	3.02	127.780	127.250	−0.41	
30	1	2.20	2.12	103.975	99.360	−4.44	
33	1	2.38	2.34	105.095	94.755	−9.84	−8.11
37	1	1.90	1.90	75.515	67.930	−10.05	
8	2	2.92	1.84	74.130	56.680	−23.53	
32	2	2.76	2.82	133.265	102.690	−22.93	−19.30
34	2	2.28	2.22	102.190	87.770	−14.11	
35	2	2.04	1.90	79.850	66.575	−16.62	
29	3	2.86	2.78	123.100	91.120	−25.98	
27	3	2.32	1.98	88.520	62.685	−29.19	−28.30
31	3	2.46	2.34	92.355	64.900	−29.73	
22	4	3.44	3.34	160.480	108.725	−32.25	
4	4	4.00	3.60	161.492	110.282	−31.72	−32.95
17	4	2.16	1.82	65.145	40.530	−23.97	
19	4	2.60	2.14	101.920	67.880	−33.41	
14	5	2.34	1.90	86.871	54.331	−37.44	
23	5	2.66	2.52	130.560	82.640	−36.71	−37.03
25	5	2.42	2.30	113.375	71.490	−36.94	
24	6	3.02	2.82	148.670	53.910	(−63.73)	
21	6	2.38	2.58	124.570	87.305	−29.92	−37.06
26	6	2.18	2.30	123.430	68.880	−44.20	
15	7	2.14	1.94	85.870	56.890	−33.75	
20	7	2.80	3.08	131.255	71.975	−45.16	−39.45
11	8	3.84	3.00	132.119	97.409	−26.27	
10	8	2.44	2.06	74.170	46.055	−37.91	−39.14
18	8	2.18	2.40	107.040	63.830	−40.36	
3	9	1.80	1.64	80.855	41.741	−48.38	
7	9	2.70	2.56	124.210	59.970	−51.72	−50.05
1	10	1.80	1.92	95.360	49.985	−37.10	
2	10	1.92	2.10	106.596	47.243	−55.68	−46.39

Loss of Weight in Atrophy.

In Table II are shown the differences between control and denervated muscles at the end of the designated period for atrophy. In general, we find a steady loss of weight with increasing time. The individual variations in each group of animals sacrificed are large, but the average figure for the group is significant. It is particularly interesting to note a loss of 55 per cent in weight through prolonged atrophy, while prolonged autolysis of normal muscle never causes more than 15 per cent hydrolysis of the protein to amino acid. In Table III are summarized the ranges of variation within each group of rabbits.

TABLE III.

Range of Individual Variations in Experimental Atrophies.

Time after denervation.	Range of variation of loss of weight.	Difference.
wks.	*per cent*	*per cent*
1	0.41–10.05	9.54
2	14.11–23.53	9.42
3	25.98–29.73	3.75
4	23.97–33.41	9.44
5	36.71–37.44	0.73
6	29.92–63.73	33.81
7	33.75–45.16	11.39
8	26.27–40.36	14.09
9	48.38–51.72	3.38
10	37.10–55.68	18.58

The curve of weight loss is of the logarithmic type (Fig. 1), ascending most rapidly at first and tending to approach an equilibrium figure. In general it resembles the curves of autolysis.

It suggests that the atrophic process eventually reaches a maximum figure, beyond which no further losses develop. The remaining tissue is obviously non-digestible and probably this is largely connective in character. The outstanding difference between this process in the body and autolysis, is one of degree. In the complete removals described by Brooks we have a still more striking difference, for which muscle autolysis *in vitro* offers no explanation.

Water Content.

Muscles from operated or normal legs were compared after drying to constant weight at 105°C. (See Table IV.)

The results indicate very little change in water content during atrophy. The fact that the differences are so small and occur in both directions makes it improbable that there is any actual increase in protein hydration in atrophy.

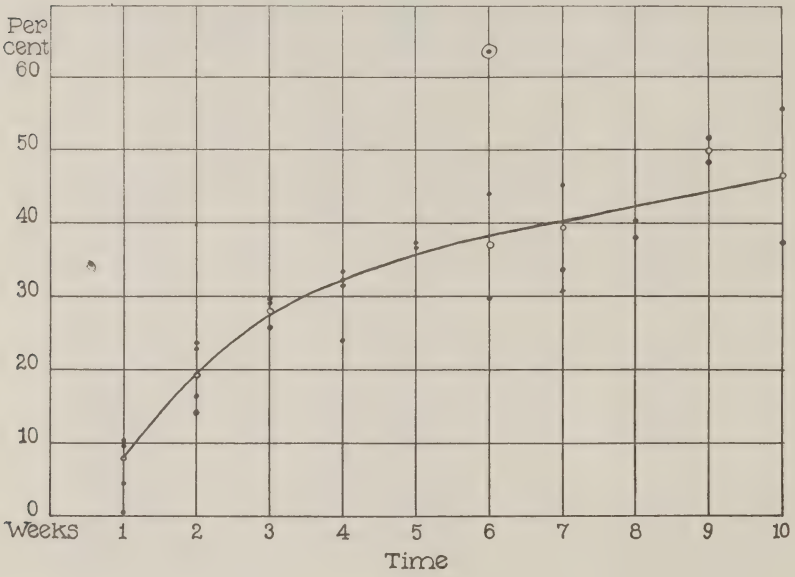


Fig. 1.

Autolysis of Normal and Atrophic Muscles.

We have made comparisons of the autolytic behavior of normal and atrophic muscles, using the technique previously described (2). Digests were set up and kept at 37°C., the amino acids being titrated in trichloroacetic acid filtrates by the formol method.

Initial differences between the two groups of muscles are too small to be significant. There evidently is no accumulation of amino acids in the atrophic muscle. In the course of 10 day autolysis, however, the atrophic muscles regularly produced

TABLE IV.

Water Content of Muscles.

Rabbit No.	Duration of experiment.	H_2O content.		
		Non-operated.	Operated.	Difference from non-operated.
	wks.	*per cent*	*per cent*	*per cent*
Control.	0	74.530	74.134	−0.396
36	1	73.902	75.728	+1.826
30	1	74.662	75.686	+1.024
33	1	74.842	74.122	−0.720
32	2	74.876	76.554	+1.678
34	2	76.118	77.628	+1.510
35	2	75.814	77.516	+1.702
29	3	74.878	76.270	+1.392
27	3	75.862	77.808	+0.946
31	3	76.270	76.608	+0.338
22	4	74.296	74.800	+0.504
4	4	74.865	76.633	+1.768
19	4	75.352	76.326	+0.974
14	5	76.796	77.852	+1.383
23	5	75.640	75.880	+0.240
25	5	75.178	75.298	+0.120
24	6	75.562	73.708	−1.854
21	6	75.924	76.408	+0.484
26	6	75.974	76.424	+0.450
15	7	75.406	76.470	+1.064
20	7	73.632	74.034	+0.402
10	8	75.318	73.616	−1.702
11	8	75.504	76.504	+1.000
18	8	76.210	74.744	−1.466
3	9	75.904	77.106	+1.202
7	9	74.390	72.474	−1.916
1	10	75.940	75.618	−0.322
2	10	76.022	75.968	−0.054
Average.....		75.34	75.76	+0.42

TABLE V.

Autolysis of Control and Atrophic Muscles.

Rabbit No.	0.2 N amino acid in 25 cc. trichloroacetic acid filtrate.														
	Non-operated (left).							Operated (right).						Difference from non-operated in 10 days.	Average.
	Weeks.	0	1 day.	5 days.	10 days.	Net gain in 10 days.	Average.	0	1 day.	5 days.	10 days.	Net gain in 10 days.	Average.		
Control.	0	0.25	0.30	0.45	0.60	0.35		0.25	0.35	0.45	0.60	0.35		0	
36	1	0.30	0.40	0.60	0.60	0.30		0.30	0.40	0.45	0.80	0.50		+0.20	
30	1	0.30	0.40	0.45	0.50	0.20	0.25	0.30	0.50	0.50	0.80	0.50	0.50	+0.30	+0.25
33	1	0.30	0.35	0.40				0.30	0.40	0.45					
32	2	0.25	0.40	0.40	0.45	0.20		0.30	0.40	0.50	0.65	0.35		+0.15	
34	2	0.25	0.30	0.55	0.55	0.30	0.25	0.30	0.30	0.70	0.90	0.60	0.48	+0.30	+0.23
35	2	0.25	0.30	0.40	0.50	0.25		0.30	0.45	0.75	0.80	0.50		+0.25	
29	3	0.25	0.40	0.50	0.60	0.35		0.30	0.45	0.75	0.90	0.60		+0.25	
27	3	0.30	0.35	0.45	0.55	0.25	0.33		0.45	0.60	0.90	0.60	0.63	+0.35	+0.30
31	3	0.20	0.30	0.55	0.60	0.40		0.30	0.40	0.60	1.00	0.70		+0.30	
22	4	0.30	0.35	0.40	0.45	0.15		0.30	0.35	0.45	0.65	0.35		+0.20	
4	4	0.30	0.35	0.45	0.50	0.20	0.25	0.25	0.40	0.55	0.85	0.60	0.55	+0.40	+0.32
19	4	0.30	0.35	0.50	0.65	0.35		0.30	0.50	0.85	1.00	0.70		+0.35	
14	5	0.20	0.30	0.65						1.05					
23	5	0.30	0.45	0.50	0.60	0.30	0.30	0.30	0.55	0.65	0.80	0.50	0.60	+0.20	+0.30
25	5	0.25	0.40	0.50	0.55	0.30		0.25	0.50		0.95	0.70		+0.40	

24	6	0.25	0.40	0.50	0.60	0.35		0.25		0.65	0.95	0.70		+0.35	+0.28
21	6	0.25	0.40	0.55	0.65	0.40	0.42	0.25	0.40	1.00	1.00	0.75	0.70	+0.35	
26	6	0.30		0.65	0.80	0.50		0.30		0.70	0.95	0.65		+0.15	
15	7	0.35	0.45	0.55	0.55	0.20		0.35	0.50	0.75	0.75	0.40		+0.20	+0.25
20	7	0.20	0.35	0.40	0.70	0.50	0.35	0.20	0.45	0.75	1.00	0.80	0.60	+0.30	
11	8	0.35	0.40	0.45				0.35	0.50	0.60					
10	8	0.25	0.30	0.35	0.45	0.20	0.27	0.25		0.55	0.75	0.50	0.55	+0.30	+0.27
18	8	0.20	0.30	0.35	0.55	0.35		0.20	0.30	0.60	0.80	0.60		+0.25	
3	9	0.25	0.30	0.35	0.35	0.10		0.20		0.60	0.80	0.60		+0.50	+0.50
7	9	0.25	0.30	0.45	0.50	0.25	0.17	0.25	0.35	0.65	1.00	0.75	0.67	+0.50	
1	10	0.25	0.30	0.35	0.45	0.20		0.25	0.30	0.45	0.65	0.40		+0.20	+0.25
2	10	0.20	0.30	0.30	0.45	0.25	0.23			0.35	0.75	0.55	0.47	+0.30	
Average.....		0.26			0.55	0.29		0.27		-	0.84	0.57		+0.30	

more amino acid than the normal, and this in spite of the fact that the atrophic muscles *in vivo* were constantly losing digestible protein. There is no significant change in the relation of normal to atrophic muscle with increased length of atrophy in the body. The difference in favor of the digestion of the atrophic muscle is rather a constant difference, and amounts to nearly 100 per cent greater than the normals (Table V).

This increased digestibility of the atrophic muscles is apparently not correlated with a measureable increase in the pH developed post mortem. In Table VI is shown the summary of pH values obtained as soon after removal, grinding, and making up the muscle digests as possible, and again after 24 hours incubation. The initial differences are found in both directions, and between averages are too small to be significant. After the more perfect equilibration of the digests 24 hours old, there is no difference between average pH figures.

TABLE VI.

Summary of pH Values Obtained from Control and Atrophic Muscle Breis. Atrophies Were of 1 to 10 Weeks Duration.

	pH: initial value.	pH: after 24 hrs. autolysis.
Control	5.79	5.95
Atrophic	5.81	5.95
Difference	0.02	0.00

If the muscle breis are acidified alike, it is found that the atrophic muscle again gives a somewhat larger amino acid production than the normal. More protein is present in atrophic muscle which is made available with added acid than in the normal tissue (Table VII).

Again, the pH values in the normal and atrophic tissues after acid is added are nearly identical (Table VIII).

If to the muscle breis alkali is added, we find again that inhibition of autolysis is greater in the normal than in the atrophic samples, although the pH values appear to favor digestion in the normal rather than in the atrophied muscle digests, if there is any difference between them. (See Tables IX and X.)

If the tyrosine reaction is developed in the trichloroacetic acid filtrates from the autolyses tabulated above we find additional

TABLE VII.

Summary of Data on Autolysis of Control and Atrophic Muscle Breis Made Acid with 25 Cc. of 0.2 N HCl per 50 gm. of Tissue. Atrophies Were of 1 to 10 Weeks Duration.

	0.2 N amino acid.			
	0	10 days.	Net gain.	No. of experiments.
	cc.	*cc.*	*cc.*	
Control	0.26	1.08	0.82	27
Atrophic	0.27	1.57	1.30	27
Difference	0.01	0.49	0.48	

TABLE VIII.

Summary of pH Values Obtained from Control and Atrophic Muscle Breis to Which 25 Cc. of 0.2 N HCl Were Added per 50 Gm. of Tissue. Atrophies Were from 1 to 9 Weeks Duration.

	pH: initial value.	pH: after 24 hrs. autolysis.	No. of experiments.
Control	4.19	4.43	20
Atrophic	4.04	4.34	20
Difference	−0.15	−0.09	

TABLE IX.

Summary of Autolytic Digests Made Alkaline by Adding 10 Cc. of 0.2 N NaOH per 50 Gm. of Muscle Tissue.

	0.2 N amino acids.		
	Initial.	10 days.	Net gain.
	cc.	*cc.*	*cc.*
Control	0.26	0.40	0.14
Atrophic	0.27	0.69	0.42
Difference	0.01	0.29	0.28

TABLE X.

Summary of pH Values Obtained from Control and Atrophic Muscle Breis to Each of Which Were Added 10 Cc. of 0.2 N NaOH per 50 Gm. of Muscle Tissue.

	Time after operation.	pH: initial value.	pH: after 24 hrs. autolysis.	No. of experiments.
	wks.			
Control		6.73	6.64	18
Atrophic	1–10	6.87	6.69	18
Difference		0.14	0.05	

evidence of greater primary protein cleavage in the atrophic muscle than in the normal. The figures also show increased cleavage in the acid brei over the normals and less inhibition in the alkaline. In all cases, digestion is greater in muscles that are undergoing atrophy. It is noteworthy that more tyrosine-reacting fragments are found in the initial samples of atrophic muscle, which seems to indicate a greater amount of primary protein fragmentation in the atrophying tissue than in the normals. (See Table XI.)

Inorganic Phosphorus.

The inorganic phosphorus was determined in two digests by the Briggs (21) modification of the Bell-Doisy method. In these cases the atrophied muscle showed less P_2O_5 than the controls. More P_2O_5 is liberated in the alkaline breis than in acid or control reaction, as was found by Sevringhaus in the case of liver (22). (Compare Table XII.)

Histological Changes.

The microscopical changes may be briefly summarized. They are not new, but serve to identify the chemical changes we have found with atrophy as described morphologically.

The longer the atrophic period the greater is the increase of fibrous tissue, adiposity, and nuclei in typical fields. Whether there is an actual increase in nuclear material, or merely a concentration in the fibers as they diminish in mass by loss of cytoplasm we cannot say. The fibers after prolonged atrophy are literally crowded with nuclei.

In only three of twenty-seven cases examined was there evidence of lymphocytosis. In two there was myositis. There seems to be little evident relation between these conditions and the extent of autolysis, or loss of weight.

In the atrophic muscle the striations were found to persist even after the 10 week period. In normal and atrophic muscles, hashed and allowed to autolyze, the striations were also clearly visible in shreds of tissue at the end of 10 days. The nuclei were also visible and stained normally.

TABLE XI.

Summary of Data on Liberation of Tyrosine in Autolysis of Control and Atrophic Muscles.

	Tyrosine per 100 gm. muscle tissue.		
	0	10 days.	Net gain.
	mg.	*mg.*	*mg.*
Control	20	69	40
Atrophic	35	116	81
Difference	15	47	41
Control + acid	29	286	257
Atrophic + "	35	311	276
Difference	6	25	19
Control + alkali	29	41	12
Atrophic + "	35	86	51
Difference	6	45	39

TABLE XII.

Summary of Data on Phosphate Liberated in the Autolysis of Control and Atrophic Muscles.

	Inorganic P per 100 gm.			No. of experiments.
	0	10 days.	Net gain.	
	mg.	*mg.*	*mg.*	
Control	160.8	200.0	39.2	4
Atrophic	140.0	170.5	30.5	4
Difference	20.8	29.5	8.7	
Control + acid	160.8	190.0	29.2	3
Atrophic + "	140.0	144.0	4.0	2
Difference	20.8	46.0	25.2	
Control + alkali	160.8	206.3	45.5	3
Atrophic + "	140.0	175.6	35.6	3
Difference	20.8	30.7	9.9	

DISCUSSION.

Following section of the nerves supplying the muscles of the rabbit's hind leg, we have found the expected paralysis, accompanied by a progressive loss of weight. At the end of 2 weeks the denervated muscles have lost 19 per cent of their weight, 50 per cent at the end of 9 weeks. This is in harmony with the observations of Langley and Kato (23), and Audova, though we believe our technique is somewhat more reliable than theirs.

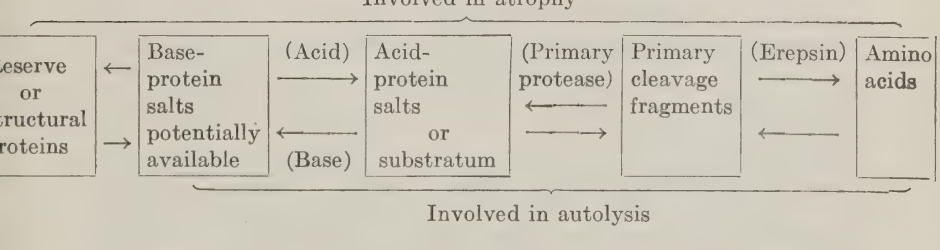
Comparing the loss of tissue in atrophy, with digestion by autolysis as measured by amino acids and as checked by the gross appearance of autolyzing muscle, we are at once impressed with the small extend of autolysis, as compared with atrophy. In 2 weeks the living muscle loses 20 per cent of its mass in atrophy. In the same period normal muscle tissue digests less than 10 per cent of its proteins to amino acid under the most favorable H ion concentration. This figure seldom goes above 15 per cent after prolonged digestion. In the same time the living muscle may lose 50 per cent or more of its protein mass. In cases studied by Brooks, the entire muscle disappeared within a few weeks, and was represented only by connective tissue.

This difference between autolytic digestion of muscle proteins, and atrophic digestion and removal, constitutes, we believe, the most striking and important point developed in this study. It will be recalled that liver and other glandular tissues, such as thymus and kidney, autolyze completely under optimum pH conditions, so that all the cell proteins liquefy and leave only a small residue which appears to consist of connective tissue at the end. While the proteins are not converted completely into amino acids, they do, however, undergo primary cleavage, and a very much larger percentage of the tissue appears finally as amino acid. At the end of 2 weeks autolysis, muscle fibers still retain their characteristic striations and are microscopically not extensively liquefied.

In the case of liver or kidney, addition of sufficient acid to convert the base-proteins into acid-protein salts is apparently all that is required to produce complete liquefaction or primary cleavage. This does not hold for muscle. There appears to be left in muscle tissue a large mass of proteins which cannot be disintegrated by the enzymes present at the moment of re-

moval. On the other hand, this same muscle left *in situ* and alive will lose 50 per cent or more of its protein mass in 10 weeks and at the end of that time autolyze even more extensively than the original muscle. There would appear, therefore, to be a mechanism involved in muscle atrophy which does not function in autolysis. The striking constancy of the autolysis figures, from muscle after atrophy for 1 to 10 weeks, suggests an equilibrium between the mass of unavailable proteins, possibly structural in function, and the mass of potentially available proteins; *i.e.*, those which are digested when converted into acid-protein salts. It will be recalled that Steyrer (14) believed there was in atrophy a progressive change of the myosin, or albumin fraction, into myogen, a globulin-like protein which digests. It was also pointed out by Jacoby (24) that in autolysis of liver the globulin disappeared while the albumin remained undigested. Acid converts this albumin into substratum in liver, but not in muscle.

From our study of muscle atrophy we have not as yet developed the nature of the mechanism by which the wholly unavailable proteins are gradually converted into potentially available substratum. It may be catalyzed by outside enzymes, such as those derived from lymphocytes. The histological evidence, however, lends little support to the idea, for in the great majority of sections of atrophic muscle examined, there was no evidence of lymphocytic invasion. Without at this time attempting an explanation of the mechanism involved, we believe that the relations within the muscle cell can be diagramatically expressed as follows:



In normal muscle the great bulk of the tissue exists in the first form, the "reserve or structural" group. A smaller fraction exists as base-protein salts, which when acidified are fragmented

by the primary protease present and eventually yield amino acids. When muscle dies, sufficient acidity develops to allow 5 per cent of the total protein to hydrolyze to the amino acid stage. By adding additional acid, this figure may be increased to 10 or 15 per cent of the total protein. We have not studied the primary cleavage products sufficiently to determine whether they are in equilibrium with the amino acids or not in muscle autolysis, but evidence which we have obtained from liver, makes this probable. Where the amino acids are removed, as in the living tissue, the primary fragments would undoubtedly be further digested, diffuse out of the cell, and disappear.

The relation of atrophic changes to muscular strength has been touched upon in a previous paper (2). It is well known that in disuse, atrophic muscles lose a large part of their contractile strength. Roberts (25) observed that after prolonged atrophy denervated muscle was indifferent to polarity in galvanic stimulation, and to strength of current as well. In the early weeks the muscle showed increased excitability. He also found that after long atrophy the denervated muscle failed to respond to faradic stimulation. Bourguignon (26) observed that when partial degeneration took place a muscle might give either a rapid or a slow contraction. It had two chronaxias, one for rapid contraction never greater than fifteen times the normal, and the other for slow contraction at least fifty times the normal. When degeneration was complete, only the slow type of contraction could be excited. It is evident that loss of strength goes on rather slowly and bears a definite relation to the extent of atrophy. When it has gone on for 10 weeks, there is probably very little contractile power left. Where the nerve regenerates, the atrophied muscle increases in mass, and contractile power is regained. The essential machinery of the muscle has not disappeared, and hypertrophy, the reversal of the atrophic change, goes on.

From the present meager data, it would appear that myogen is the protein most concerned in contraction, and when it is converted into myosin, fragmented and digested, contraction is impossible.

During atrophy the water content of muscle shows little or no increase. The pH values also are so close to normal that there

is no certain indication of increased acid-protein in the atrophic muscle. Autolysis, however, shows an increased amount of acid-protein salt, and also of the potentially available protein fraction.

Postmortem H ion changes are the same for both normal and atrophied muscles, which suggests also that the acid production in the atrophied and normal muscle is in direct proportion to the mass of muscle proteins in each.

Histologically all our tissues, except one, show distinct striations, cross and longitudinal. The microscopical evidence is strong confirmation of the chemical evidence that the integrity of muscle structure persists under extreme conditions.

The multiplication of nuclei in atrophic fibers is striking and characteristic. This may be merely a relative concentration of material which does not decrease in conditions such as those found in these experiments. We have made no determinations of nuclear material and cannot, therefore, assume proliferation of nuclear substance.

The increase of connective and adipose tissue is, we believe, a compensatory invasion from intermuscular septa and is a secondary phenomenon to atrophy of the muscle itself.

Langley and Kato (23) believe that fibrillation is the first step in atrophy. In frogs they found no fibrillation and little atrophy. In other muscles fibrillation and atrophy were observed. They also found increased oxygen consumption, indicating increased breakdown. Our own observations neither confirm nor disprove this mechanism.

SUMMARY.

1. After section of the femoral and sciatic nerves in rabbits, the muscles undergo rapid atrophy at first, the rate of weight loss gradually diminishing. The curve shows an average loss of 19 per cent in weight in 2 weeks, 50 per cent in 9 weeks.

2. The atrophied muscle shows no definite increase of water content.

3. In postmortem autolysis, lasting 10 days, the atrophic muscles liberated more amino acids than the normal. This increase in the mass of available proteins is as great at the end of 9 weeks as at the end of 1 or 2. It is believed to represent a

gradual progressive alteration in the mass of cell proteins in which the unavailable structural proteins are converted into digestible forms.

4. Atrophic muscle contains no more free amino acids than normal muscle. It does contain a little more of the primary cleavage products, as shown by the reaction for tyrosine peptides.

5. It is suggested that there is an equilibrium in the cell between the non-available cell proteins and the type which becomes available by the addition of acid.

6. Atrophied muscle appears to lose less phosphorus than the normal in autolysis. When connective tissue is excluded, however, this difference is not significant.

7. Atrophic and normal muscles develop the same postmortem pH. There is no measureable increase in acidity in the muscle cell undergoing atrophy. Because of the alteration in protein content, the atrophic muscle has lost a small amount of buffer capacity against both acid and alkali.

8. Histologically the atrophic muscle fibers retain their striæ, and these persist like the normals, after 10 days of autolysis. The atrophied muscle fibers are found shrunken and smaller than the normal, with a relatively larger number of nuclei present, and with increased connective and adipose tissues—whether absolute or merely relative was not determined.

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THE BLOOD ELECTROLYTE CHANGES IN ETHER ACIDOSIS.

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In the study of the changes in the acid-base balance of the blood as affected by ether anesthesia Van Slyke, Austin, and Cullen (1922) and Cullen, Austin, Kornblum, and Robinson (1923) have shown that there is a true acidosis with fall in both the pH and the alkali reserve of the blood and that this develops with great rapidity. No evidence was obtained, however, which permitted conclusions as to the relative importance of withdrawal of base from the blood, on the one hand, or of introduction of acid into the blood on the other hand.

Leake, Leake, and Koehler (1923), in similar studies, observed some increase in the acetone bodies in the blood in short anesthesias, but the increase observed was never sufficient to account for the fall in alkali reserve. They concluded:

> "Other acid products may be found, of course, but this is unlikely, since the acetone bodies seem to be the typical non-volatile acids produced in disturbed metabolic conditions, especially when any interference with oxidative processes takes place.
>
> "The evidence indicates that the acidosis of ether anesthesia can be explained neither by a compensatory reaction to an acapnia, nor by entrance of acid into the blood, but rather by the withdrawal of base from it."

The studies of Barr and his associates (1923) suggest the possible importance of lactic acid as a cause for fall in alkali reserve. The studies here described were made to throw further light upon the

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relative importance of removal of base and of introduction of acid into the blood in causing the fall in alkali reserve in these experiments.

In addition to the analyses employed in our former studies (CO_2 content and pH of serum and CO_2 content and O_2 content and capacity of blood), we investigated the total bases, the chlorides, the protein content, the water content, and the specific gravity of the blood and serum and the conductivity and freezing point depression of the serum.

Methods.

Animals.—In order to obtain large samples of blood (120 cc.) we used large dogs, weighing more than 20 kilos, for the experiments.

Bleeding.—The blood was drawn from the left ventricle of the heart through a No. 16 lumbar puncture needle into tubes under oil. It was then defibrinated in the manner described by Austin and Gram (1924). Immediately afterwards the blood was divided into a portion for centrifuging and another portion for whole blood analysis. The first portion was centrifuged at once with the usual precautions (Cullen, Austin, Kornblum, and Robinson) against loss of CO_2, while the latter was kept in a tube surrounded by crushed ice until used. The serum after separation was transferred to Haldane sampling tubes over mercury.

Anesthesia.—The anesthesia was produced by covering the muzzle with a towel saturated with ether until complete relaxation ensued and continued by the drop method upon a few layers of gauze over the muzzle. The total duration of anesthesia in these experiments was 20 minutes.

Measurements and Analytical Methods.

Hydrogen Ion Concentration.—The pH of serum was measured colorimetrically by the method of Cullen (1922) at room temperature, using the correction -0.34 to convert colorimeter pH 20° to true pH 38°. For dog serum probably the correction 0.30 is closer to the average value than 0.34 which had been established previously for dog plasma. The use of the lower figure would make, however, no significant alteration in the calculated changes here given.

Carbon Dioxide.—(CO_2) was determined by Van Slyke's method, using the constant volume apparatus (Van Slyke and Neill (1924)). The calculation of ($BHCO_3$) from the determined total (CO_2) and pH at 38°C. was based upon Hasselbalch's equation

$$pH = pK_1 + \log \frac{(BHCO_3)}{(H_2CO_3)}$$

assuming a pK_1 of 6.10 and Bohr's solubility coefficients for serum and blood.

Chlorides.—(Cl) was determined in blood and serum by the method of Van Slyke (1923).

Cell Volumes.—The cell volumes were determined by the use of special capillary hematocrit tubes about 10 cm. long and of 0.5 mm. bore. These tubes were carefully calibrated. After filling the tubes in triplicate they were closed at both ends by a heavy rubber band and packed tightly together in the same centrifuge cup. The samples from all the bleedings of any one experiment were centrifuged simultaneously in the electric centrifuge until complete transparency resulted. Usually 1 hour at 2,500 to 3,000 R.P.M. was sufficient. The variations in the triplicate readings from each bleeding were within 0.5 per cent of the mean.

Conductivity.—Conductivity of serum was determined by the Kohlrausch bridge and expressed in mM per l. of NaCl equivalents at 20°C.

The conductivity observed was corrected for the presence of protein by the formula (Gram and Cullen (1923)).

$$C_c = C_o . 100 \frac{100}{100-2.2p}$$

C_c = corrected NaCl equivalent of conductivity.
C_o = observed " " " "
p = protein gm. per 100 cc.

Serum Protein.—This was determined by the refractometer at 17.5°C., using Reiss' figures for converting refractive index into grams of protein per 100 cc.

Oxygen Capacity.—The oxygen capacity was determined colorimetrically with an Autenrieth colorimeter standardized against

the oxygen capacity of dog blood determined by the method of Van Slyke and Stadie.

Oxygen Content.—The oxygen content was determined by the technique of Van Slyke and Stadie (1921), using the original Van Slyke apparatus.

Freezing Point.—The freezing point of serum was determined by the Burian and Drucker (1909–10) instrument with controls of conductivity water. The results were expressed in mM per l. of NaCl equivalent.

Total Base.—In determining the total base of blood and serum the base was ashed and changed to the sulfates by the total base method of Van Slyke, Wu, and McLean (1923). The sulfate was then determined by a modification of the benzidine method which will be reported by Cullen and Robinson.

Water Content.—The water content was determined by drying to constant weight at 110°C. about 2 cc. of either blood or serum. A weighing bottle was prepared with cylindrical fluted filter paper, and the fluid was run onto the filter paper. This prevented caking in the bottom of the bottle.

The observed values in gm. per 1,000 gm. are multiplied by the specific gravity and stated in the tables as (H_2O) gm. per 1,000 cc.

Specific Gravity.—The specific gravity was determined by the pycnometer method, using 2 cc. pycnometers.

Notation.—In the tables and text symbols in parentheses indicate concentration per liter of serum, cells, or blood in millimols per liter, mM per l.; symbols in brackets indicate concentration per kilo of water in serum, cells, or blood.

To approximate the base bound by protein we have applied the formulas developed by Van Slyke, Wu, and McLean (1923) from horse blood, using our observed data for protein of serum, hemoglobin, oxygenated and unoxygenated, and pH of serum. To estimate the pH_c we have assumed, following Van Slyke, Wu, and McLean:

$$\log\left[\frac{[BHCO_3]_s}{[BHCO_3]_c}\right] = \log r = pH_s - pH_c$$

For base bound by protein in cells we have used

$$(BP)_c = 3.35\ (Hb)_c\ (pH_c - 6.74) + (O_2)_c\ (0.25\ pH_c - 1.18)^*$$

where $(Hb)_c$ = oxygen capacity in mM, $(O_2)_c$ = oxygen content in mM, and $(BP)_c$ = base bound by protein in milli-equivalents.

*Van Slyke, Wu, and McLean (1923), p. 811.

TABLE I.

Experiment 38.

Serum per 1,000 cc.

Sample No.	pH colorimetric 38°	Assumed pK_1.	(CO_2)	(H_2CO_3)	p_{CO_2}	$(BHCO_3)$	(Cl)	(H_2O)	Specific gravity.	Protein.	Cond. NaCl equivalent.	Corrected cond. NaCl equivalent.	Freezing point NaCl equivalent.	(B protein) change.
			*m*M	*m*M	*mm.*	*m*M	*m*M	*gm.*		*gm.*	*m*M	*m*M	*m*M	*m*M
1	7.37	6.10	22.13	1.13	36	21.00	113.1	943	1.0215	67	117.7	138.1	172.1	
2	7.07	6.10	14.84	1.43	45	13.41	116.5	941	1.0200	66	119.1	139.2	173.5	−0.15

Blood per 1,000 cc.

Sample No.	O_2 capacity.	O_2 unsaturation.	(CO_2)	(H_2CO_3)	p_{CO_2}	$(BHCO_3)$	(Cl)	(H_2O)	Specific gravity.	Cell volume.	(B protein) change.
	*m*M	*m*M	*m*M	*m*M	*mm.*	*m*M	*m*M	*gm.*		*cc.*	*m*M
1	8.88	0.32	18.25	1.07	36	17.18	89.4	832	1.055	475	
2	8.79	1.43	13.07	1.35	45	11.72	93.4	836	1.055	467	−8.9

Cells per 1,000 cc.

Sample No.	(Hb)	(CO_2)	(H_2CO_3)	$(BHCO_3)$	(Cl)	(H_2O)	(B protein) change.
	gm.	*m*M	*m*M	*m*M	*m*M	*gm.*	*m*M
1	313	13.95	1.00	12.95	63.2	710	
2	314	11.05	1.26	9.79	67.0	715	−16.8

Per kilo serum H_2O.			Per kilo cell H_2O.		Calculation.		
Sample No.	$[BHCO_3]$	[Cl]	$[BHCO_3]$	[Cl]	$r = \frac{[BHCO_3]_s}{[BHCO_3]_c}$	$\log r = pH_s - pH_c$	pH_c
	*m*M	*m*M	*m*M	*m*M			
1	22.28	120.0	18.24	89.0	1.221	0.09	7.28
2	14.25	123.8	13.70	93.7	1.040	0.02	7.05

TABLE II.

Experiment 39.

Serum per 1,000 cc.

Sample No.	pH colorimetric 38°	Assumed pK_1.	(CO_2)	(H_2CO_3)	p_{CO_2}	$(BHCO_3)$	(Cl)	(B)	(H_2O)	Specific gravity.	Protein.	Cond. NaCl equivalent.	Corrected cond. NaCl equivalent.	Freezing point NaCl equivalent.	(B protein) change.
			*m*M	*m*M	*mm.*	*m*M	*m*M	*m*M	*gm.*		*gm.*	*m*M	*m*M	*m*M	*m*M
1	7.35	6.10	28.55	1.52	48	27.03	108.7	166.3	944	1.021	66	120	141	172	
2	7.22	6.10	19.45	1.37	43	18.08	111.2	162.3	940	1.021	70	121	143	169	+0.1

Blood per 1,000 cc.

Sample No.	O_2 capacity.	O_2 unsaturation.	(CO_2)	(H_2CO_3)	p_{CO_2}	$(BHCO_3)$	(Cl)	(B)	(H_2O)	Specific gravity.	Cell volume.	(B protein) change.
	*m*M	*m*M	*m*M	*m*M	*mm.*	*m*M	*m*M	*m*M	*gm.*		*cc.*	*m*M
1	7.90	1.38	23.43	1.44	48	21.99	85.4	152.5	844	1.052	429	
2	8.11	1.55	16.49	1.29	43	15.20	87.9	149.4	838	1.054	440	−1.8

Cells per 1,000 cc.

Sample No.	(Hb)	(CO_2)	(H_2CO_3)	$(BHCO_3)$	(Cl)	(B)	(H_2O)	(B protein) change.
	gm.	*m*M	*m*M	*m*M	*m*M	*m*M	*gm.*	*m*M
1	308	16.57	1.33	15.24	54.2	133.9	710	
2	308	12.72	1.18	11.54	58.0	132.8	708	−4.9

	Per kilo serum H_2O.			Per kilo cell H_2O.			Calculation.		
Sample No.	$[BHCO_3]$	[Cl]	[B]	$[BHCO_3]$	[Cl]	[B]	$r = \frac{[BHCO_3]_s}{[BHCO_3]_c}$	$\log r = pH_s - pH_c$	pH_c
	*m*M	*m*M	*m*M	*m*M	*m*M	*m*M			
1	28.64	115.1	176.2	21.47	76.4	188.6	1.335	0.13	7.22
2	19.23	118.2	172.7	16.30	81.9	187.7	1.180	0.07	7.15

TABLE III.

Experiment 40.

Serum per 1,000 cc.

Sample No.	pH colorimetric 38°	Assumed pK_1.	(CO_2)	(H_2CO_3)	p_{CO_2}	$(BHCO_3)$	(Cl)	(B)	(H_2O)	Specific gravity.	Protein.	Cond. NaCl equivalent.	Correct cond. NaCl equivalent.	Freezing point NaCl equivalent.	(B protein) change.
			*m*M	*m*M	*mm.*	*m*M	*m*M	*m*M	*gm.*		*gm.*	*m*M	*m*M	*m*M	*m*M
1	7.34	6.10	21.81	1.19	38	20.62	108.1	178.3	940	1.0205	70	125	148	172.0	
2	7.17	6.10	16.00	1.25	39	14.75	114.0	175.6	939	1.0200	69	126	149	175.1	−1.0

Blood per 1,000 cc.

Sample No.	O_2 capacity.	O_2 unsaturation.	(CO_2)	(H_2CO_3)	p_{CO_2}	$(BHCO_3)$	(Cl)	(B)	(H_2O)	Specific gravity.	Cell volume.	(B protein) change.
	*m*M	*m*M	*m*M	*m*M	*mm.*	*m*M	*m*M	*m*M	*gm.*		*cc.*	*m*M
1	8.56	0.00	17.67	1.12	38	16.55	83.3	161.3	827	1.055	496	
2	8.35	1.80	13.24	1.18	39	12.06	85.8	159.6	826	1.055	492	−6.6

Cells per 1,000 cc.

Sample No.	(Hb)	(CO_2)	(H_2CO_3)	$(BHCO_3)$	(Cl)	(B)	(H_2O)	(B protein) change.
	gm.	*m*M	*m*M	*m*M	*m*M	*m*M	*gm.*	*m*M
1	288	13.45	1.05	12.40	58.1	143.5	712	
2	283	10.38	1.11	9.27	56.7	142.3	709	−12.1

Per kilo serum H_2O.				Per kilo cell H_2O.			Calculation.		
Sample No.	$[BHCO_3]$	[Cl]	[B]	$[BHCO_3]$	[Cl]	[B]	$r = \frac{[BHCO_3]_s}{[BHCO_3]_c}$	$\log r = pH_s - pH_c$	pH_c
	*m*M	*m*M	*m*M	*m*M	*m*M	*m*M			
1	21.94	115.0	189.8	17.42	81.6	201.7	1.259	0.10	7.24
2	15.71	121.4	187.1	13.08	80.0	200.9	1.201	0.08	7.09

For base bound by protein in serum we have used

$$(BP)_s = 0.068\ (P)_s\ (pH_s - 4.80)\dagger$$

where $(P)_s$ = gm. of protein per liter of serum and $(BP)_s$ = base bound by protein in milli-equivalents.

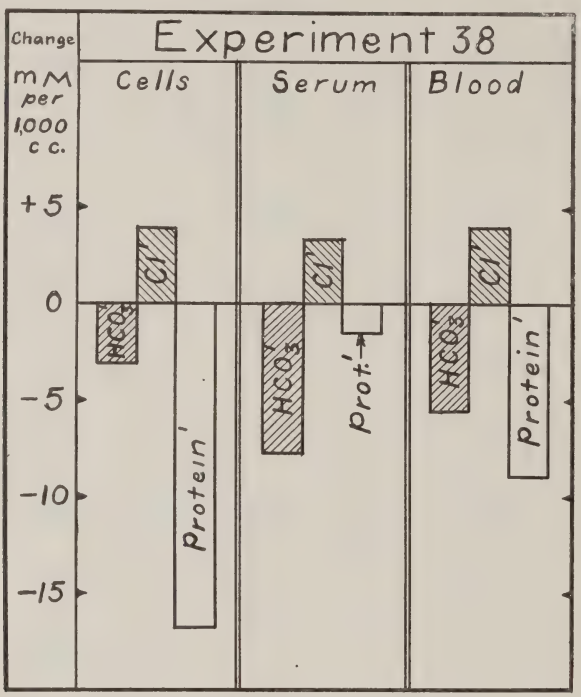

Fig. 1. Changes in electrolytes in whole blood, serum, and cells after 20 minutes anesthesia. HCO'_3 and Cl′ represent changes in those ions. Protein′ indicates the calculated change in protein equivalents of base.

For base bound by protein in blood we use

$$(BP)_B = (BP)_c \times \frac{\text{cell volume}}{1{,}000} + (BP)_s \times \left(1 - \frac{\text{cell volume}}{1{,}000}\right)$$

The results of these experiments are given in Tables I, II, and III. The changes that took place in the blood electrolytes after 20 minutes ether anesthesia are shown graphically in Figs. 1, 2, and 3.

†Van Slyke, Wu, and McLean (1923), p. 806.

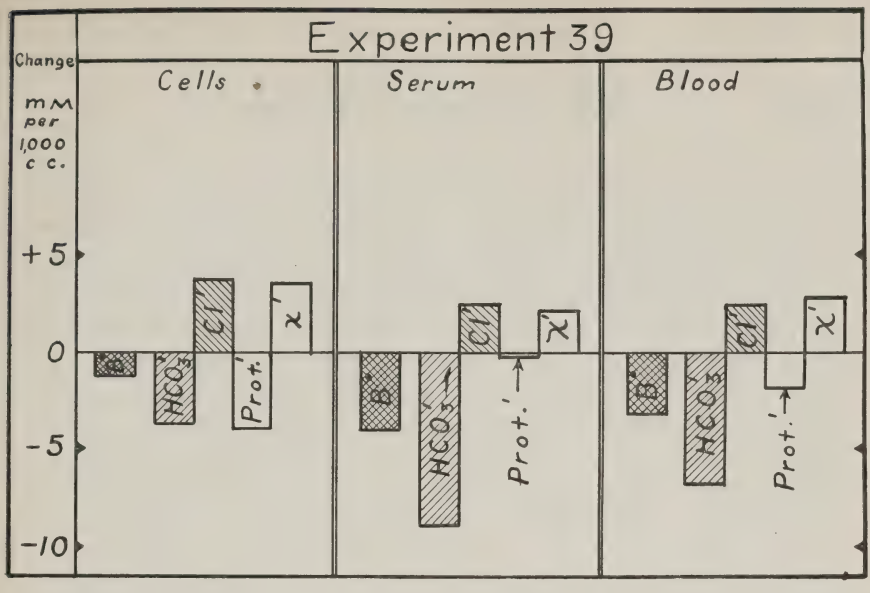

FIG. 2. Changes in electrolytes in whole blood, serum, and cells after 20 minutes anesthesia. B′ represents the change in total base as determined. Protein′ represents the calculated change in protein equivalents of base. *X*′indicates the discrepancy between the total base "B" change and the sum of the changes of HCO'_3, Cl′, and Protein.′

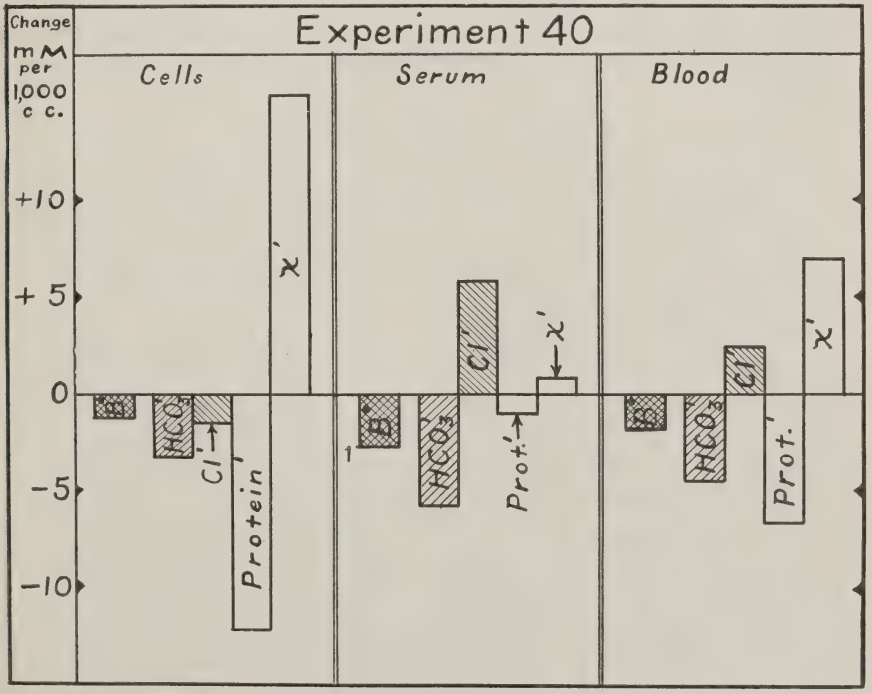

FIG. 3. Changes in electrolytes in whole blood, serum, and cells after 20 minutes anesthesia. B′ represents the change in total base as determined. Protein′ represents the calculated change in protein equivalents of base. *X*′ indicates the discrepancy between the total base "B" change and the sum of the changes of HCO'_3, Cl′, and Protein′.

DISCUSSION.

At first sight, examination of the factors investigated in the whole blood suggests that the only changes are a slight decrease in base and a moderate rise in Cl′ associated with a marked fall in HCO'_3 and that the algebraic sum of the changes in HCO'_3 and Cl′ approximately equals the change in base. Such an interpretation would give no suggestion of change in any other anion in the blood but is clearly incomplete. When it is considered that the base bound by protein is a function of pH and that we are dealing here with marked changes in pH, it becomes desirable to approximate the change in base bound by protein at the two pH levels. As an approximation we have applied the formulas developed by Van Slyke, Wu, and McLean as described above. The large decrease in BP, calculated by this method, may have a considerable quantitative error, although probably not more than 25 per cent. The algebraic sum of the changes in all anions must equal the algebraic sum of the cation change. Assuming that the percentage dissociation of the salts of the blood is the same and equating the total base to the total acid bound by base, all expressed in milli-equivalents we can write:

$$B = BHCO_3 + BCl + BP + BX$$

where X' represents one or more unidentified anions. Applying this equation to these experiments we obtain the results shown in the graphs. In view of the diminished base-binding property of protein a definite increase in one or more unidentified acids in the blood must have occurred.

This analysis of the changes observed in the whole blood in these experiments indicates that we are dealing with an introduction of Cl′ and also one or more unidentified anions X' and with an associated, quantitatively less important, withdrawal of base from the blood. If the removal of CO_2 by hyperventilation were the primary phenomenon one would observe a fall in $BHCO_3$, and the fall in base and increase in Cl′ and other anions could be considered compensatory; but this order of events would necessarily occur with an increased pH. An increased pH has never been observed in our experiments. In association with a fall in pH, the fall in $BHCO_3$ is, as has been pointed out in our previous

papers, to be considered secondary to the introduction of Cl′ and the unidentified anion or anions and to the quantitatively less important, withdrawal of base. This group of phenomena can be accounted for on the hypothesis that there is production, in some tissue or tissues, of the acid corresponding to the unidentified anion. Among the possible acids, lactic acid is to be considered; but our studies upon this point are not yet conclusive. The liberation of such an acid in the tissues may be expected to lead to a passage from the tissues to the blood of all anions for which the cells are permeable and probably somewhat in the proportions in which these anions exist in the tissues. This would account for the striking passage of Cl′ into the blood along with the unidentified anions.

The evidence of our experiments is that there is a movement of base either into the tissue fluids or out through the excretory organs. The diminished total base in the blood is associated with a greater diminution of base bound by protein. Probably to this fact is to be attributed the relative constancy of the conductivity of the serum and of the freezing point depression of the serum, for the salts of protein have probably less influence per equivalent on both conductivity and freezing point than have the salts of other anions. The relative constancy of conductivity and of freezing point depression suggest a rapid adjustment of the total osmotic pressure of the blood, in spite of the considerable liberation of base from protein and the introduction of acids from the tissues. This adjustment probably is a function of the excretory organs and especially of the kidney.

In Experiments 39 and 40 the respiratory center has maintained a ventilation which results in an intermediate result between constancy of CO_2 tension and constancy of pH. In Experiment 38, however, there was rise of CO_2 tension.

The blood changes would seem to be incident to the equilibrium between the tissues and blood subsequent to the production of an unidentified acid in the tissues and supplemented by a regulation of the total osmotic pressure of the blood.

It seems to us that in other experiments recently reported in the literature, where an apparently more or less complete balance in the increase of one anion and in the decrease of another is associated with a marked change in pH, a more complete analysis

of the changes that occur in the blood can be made by taking into account the change in base bound by protein and the change in total base.

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INDEX TO AUTHORS.

A

Allen, Edgar. See Doisy, Ralls, Allen, and Johnston, 711

Anderson, Arthur K., and **Schutte, Harry S.** The determination of nitrogen in connection with the wet combustion method for carbon, 57

Anderson, R. J. A contribution to the chemistry of grape pigments. III. Concerning the anthocyans in Seibel grapes, 685

— and **Nabenhauer, Fred P.** A contribution to the chemistry of grape pigments. II. Concerning the anthocyans in Clinton grapes, 97

Anderson, William E., and **Smith, Arthur H.** The effect of acute scurvy on the subsequent nutrition and growth of guinea pigs, 181

Austin, J. Harold. A note on the estimation of carbon dioxide in serum in the presence of ether by the Van Slyke method, 345

—, **Cullen, G. E., Gram, H. C.,** and **Robinson, H. W.** The blood electrolyte changes in ether acidosis, 829

B

Barr, David P. See Loebel, Barr, Tolstoi, and Himwich, 9

Baudish, Oskar, and **Welo, Lars A.** On the mechanism of the catalytic action of iron salts. I, 261

Baumann, Emil J. A simple method for preparing large quantities of yeast nucleic acid as a magnesium compound, 1

Bell, Marion. See Blatherwick, Bell, and Hill, 241

Benedict, Stanley R., and **Theis, Ruth C.** A modification of the molybdic method for the determination of inorganic phosphorus in serum, 63

—. See Nash and Benedict, 423

—. See Theis and Benedict, 67

Black, A. See Steenbock and Black, 405

Blatherwick, N. R., Bell, Marion, and Hill, Elsie. Some effects of insulin on the carbohydrate and phosphorus metabolism of normal individuals, 241

Blumenstock, Julius, and **Ickstadt, Albert.** A note on the rôle of the liver in parathyroid tetany, 91

Bodansky, Aaron. A study of a milk-coagulating enzyme of *Solanum elaeagnifolium*, 365

Borgstrom, P. See Denis and Borgstrom, 109

Bradley, H. C. See Chen, Meek, and Bradley, 807

—. See Hertzman and Bradley, 275

C

Carman, J. S. See Mattill, Carman, and Clayton, 729

Chen, K. K., Meek, Walter, and **Bradley, H. C.** Studies of autolysis. XII. Experimental atrophy of muscle tissue, 807

Chibnall, Albert Charles. Spinacin, a new protein from spinach leaves, 303

Clayton, M. M. See Mattill, Carmen, and Clayton, 729

Cohn, David J. See Nelson and Cohn, 193

Collins, Arnold M. See Jacobs and Collins, 387

Colwell, A. R. Insulin and phlorhizin glycosuria, 289

Cox, Gerald J. See Rose and Cox, 747

Cullen, G. E. See Austin, Cullen, Gram, and Robinson, 829

D

Dakin, H. D. The formation of *l*-malic acid as a product of alcoholic fermentation by yeast, 139

—. A note on the presence of valine in zein, 137

Daniels, Amy L., and **Stearns, Genevieve.** The effect of heat treatment of milk feedings on the mineral metabolism of infants, 225

Deas, J. Yeast growth-promoting vitamin tested for its effects on animals, 5

Denis, W., and **Borgstrom, P.** A study of the effect of temperature on protein intake, 109

Dienes, L. Further studies on the determination of calcium, magnesium, and phosphorus in animal substances, 77

—. A note on the gravimetric microchemical technique, 73

Doisy, Edward A., Ralls, J. O., Allen, Edgar, and **Johnston, C. G.** The extraction and some properties of an ovarian hormone, 711

Dunn, L. C. The effect of cod liver oil in various amounts and forms on the growth of young chickens, 129

E

Eaton, Emily P. See Ronzoni, Koechig, and Eaton, 465

F

Felsher, Augusta R. See Wang and Felsher, 659

Fred, E. B. See SCHMIDT, PETERSON, and FRED, 163

Friedemann, T. E. See SHAFFER and FRIEDEMANN, 585

G

Gram, H. C. Chlorides of serum, blood, and corpuscles in various pathological conditions, 337

—. See AUSTIN, CULLEN, GRAM, and ROBINSON, 829

Greenwald, Isidor. Is there a toxin in the blood of parathyroidectomized dogs? 33

—. Some chemical changes in the blood of dogs after thyroparathyroidectomy, 649

H

Haller, H. L. See LEVENE, TAYLOR, and HALLER, 157

Harington, C. R., and **Van Slyke, Donald D.** On the determination of gases in blood and other solutions by vacuum extraction and manometric measurement. II, 575

Hart, E. B. See STEENBOCK, HART, and JONES, 775

Hastings, A. Baird, and **Sendroy, Julius, Jr.** The colorimetric determination of blood pH at body temperature without buffer standards, 695

—, —, **Murray, Cecil D.,** and **Heidelberger, Michael.** Studies of gas and electrolyte equilibria in blood. VII. The effect of carbon monoxide on the acidity of hemoglobin, 317

Hawkins, James A. The acid-base equilibrium of the blood of normal guinea pigs, rabbits, and rats, 147

Heidelberger, Michael. See HASTINGS, SENDROY, MURRAY, and HEIDELBERGER, 317

Hendrix, Byron M., and **McAmis, Ava J.** Alkalosis produced by injections of hydrazine sulfate in dogs, 45

Hertzman, A. B., and **Bradley, H. C.** Studies of autolysis. XI. Relation of the isoelectric point to digestibility, 275

Hill, Elsie. See BLATHERWICK, BELL, and HILL, 241

Himwich, Harold E. See LOEBEL, BARR, TOLSTOI, and HIMWICH, 9

Hirsch, Edwin F. Changes in the hydrogen ion concentration of the blood with coagulation, 795

Howe, Paul E. The differential extraction and precipitation of the soluble proteins of muscle, with data on the concentration of proteins in the muscle of the calf, cow, and rabbit, 493

Hubbard, Roger S., and **Wright, Floyd R.** Variations in the rate of excretion of the acetone bodies during the day, 377

I

Ickstadt, Albert. See Blumenstock and Ickstadt, 91

J

Jacobs, Walter A., and **Collins, Arnold M.** Strophanthin. V. The isomerization and oxidation of isostrophanthidin, 387

Johnston, C. G. See Doisy, Ralls, Allen, and Johnston, 711

Jones, J. H. See Steenbock, Hart, and Jones, 775

K

Koechig, Irene. See Ronzoni, Koechig, and Eaton, 465

L

Leavenworth, Charles S. Note on the basic amino acids yielded by casein, 315

—. See Osborne, Leavenworth, and Nolan, 309

Leiboff, S. L. A simplified method for cholesterol determination in blood, 177

Levene, P. A., Simms, H. S., and **Pfaltz, Mimosa H.** The relation of chemical structure to the rate of hydrolysis of peptides. I. On the synthesis, on the physical constants, and on the rates of hydrolysis of methylated peptides, 445

—, **Taylor, F. A.,** and **Haller, H. L.** On lignoceric acid, 157

Levene, P. A., and **van der Hoeven, B. J. C.** The concentration of vitamin B, 429

Lewis, Howard B. See Updegraff and Lewis, 633

Loebel, Robert O., Barr, David P., Tolstoi, Edward, and **Himwich, Harold E.** Studies of the effect of exercise in diabetes. II. Lactic acid formation in phlorhizin diabetes, 9

M

McAmis, Ava J. See Hendrix and McAmis, 45

Mattill, H. A., Carman, J. S., and **Clayton, M. M.** The nutritive properties of milk. III. The effectiveness of the X substance in preventing sterility in rats on milk rations high in fat, 729

Meek, Walter. See Chen, Meek, and Bradley, 807

Murray, Cecil D. See Hastings, Sendroy, Murray, and Heidelberger, 317

N

Nabenhauer, Fred P. See Anderson and Nabenhauer, 97

Nash, Thomas P., Jr., and **Benedict, Stanley R.** On the mechanism of phlorhizin diabetes. Second paper, 423

Neill, James M. See Van Slyke and Neill, 523

Nelson, J. M., and **Cohn, David J.** Invertase in honey, 193

Nolan, Laurence S. See Osborne, Leavenworth, and Nolan, 309

O

Osborne, Thomas B., Leavenworth, Charles S., and Nolan, Laurence S. A note on Dakin's method as applied to edestin 309

P

Peterson, W. H. See Schmidt, Peterson, and Fred, 163

Petrén, Karl. Low nitrogen metabolism with low carbohydrate diet in diabetes, 355

Pfaltz, Mimosa H. See Levene, Simms, and Pfaltz, 445

Pucher, George W. See Youngburg and Pucher, 741

Q

Quick, Armand J. A method for the quantitative determination of menthol glycuronic acid in urine, 667

—. The synthesis of menthol glycuronic acid in the rabbit, 679

R

Ralls, J. O. See Doisy, Ralls, Allen, and Johnston, 711

Robinson, H. W. See Austin, Cullen, Gram, and Robinson, 829

Ronzoni, Ethel, Koechig, Irene, and Eaton, Emily P. Ether anesthesia. III. Rôle of lactic acid in the acidosis of ether anesthesia, 465

Rose, W. C., and Cox, Gerald J. The relation of arginine and histidine to growth, 747

S

Schmidt, E. G., Peterson, W. H., and Fred, E. B. The formation of *l*-leucic acid in the acetone-butyl alcohol fermentation, 163

Schutte, Harry S. See Anderson and Schutte, 57

Sendroy, Julius, Jr. See Hastings and Sendroy 695

—. See Hastings, Sendroy, Murray, and Heidelberger, 317

Shaffer, P. A., and Friedemann, T. E. Antiketogenesis. V. The ketolytic reaction; action of glycol aldehyde and of glyoxal, 575

Simms, H. S. See Levene, Simms, and Pfaltz, 445

Smith, Arthur H. See Anderson and Smith, 181

Smith, Lawrence Weld. The experimental feeding of dried breast milk, 625

Stearns, Genevieve. See Daniels and Stearns, 225

Steenbock, H., and Black, A. Fat-soluble vitamins. XVII. The induction of growth-promoting and calcifying properties in a ration by exposure to ultra-violet light, 405

Steenbock, H., Hart, E. B., and **Jones, J. H.** Fat-soluble vitamins. XVIII. Sunlight in its relation to pork production on certain restricted rations, 775

T

Taylor, F. A. See LEVENE, TAYLOR, and HALLER, 157

Theis, Ruth C., and **Benedict, Stanley R.** The determination of phenols in the blood, 67

—. See BENEDICT and THEIS, 63

Tolstoi, Edward. See LOEBEL, BARR, TOLSTOI, and HIMWICH, 9

U

Updegraff, Helen, and **Lewis, Howard B.** A quantitative study of some organic constituents of the saliva, 633

V

van der Hoeven, B. J. C. See LEVENE and VAN DER HOEVEN, 429

Van Slyke, Donald D., and **Neill, James M.** The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I, 523

Van Slyke, Donald D. See HARINGTON and VAN SLYKE, 575

Vickery, Hubert Bradford. Some nitrogenous constituents of the juice of the alfalfa plant. II. The basic nitrogen, 117

W

Wang, Chi Che, and **Felsher, Augusta R.** The effect of alcoholic extract of commercial granular glucose on urinary reducing substance, 659

Welo, Lars A. See BAUDISH and WELO, 261

Wright, Floyd R. See HUBBARD and WRIGHT, 377

Y

Youngburg, Guy E., and **Pucher, George W.** Studies on pentose metabolism. I. A colorimetric method for the estimation of furfural, 741

INDEX TO SUBJECTS.

A

Acetone:
-Butyl alcohol fermentation, *l*-leucic acid formation in (SCHMIDT, PETERSON, and FRED) 163

Acetone bodies:
Excretion, variation in rate during day (HUBBARD and WRIGHT) 377

Acid-base equilibrium:
Blood of normal guinea pigs, rabbits, and rats (HAWKINS) 147

Acidity:
Hemoglobin, effect of carbon monoxide on (HASTINGS, SENDROY, MURRAY, and HEIDELBERGER) 317

Acidosis:
Ether anesthesia, rôle of lactic acid in (RONZONI, KOECHIG, and EATON) 465
—, blood electrolyte changes in (AUSTIN, CULLEN, GRAM, and ROBINSON) 829
Studies (HASTINGS and SENDROY) 695

Alcohol:
Extract of commercial granular glucose, effect on urinary reducing substance (WANG and FELSHER) 659

Alcohol—*continued:*
Fermentation by yeast, *l*-malic acid as a product (DAKIN) 139

Alfalfa:
Plant, juice, nitrogenous constituents (VICKERY) 117

Alkalosis:
Hydrazine sulfate injections in dogs, alkalosis produced by (HENDRIX and MCAMIS) 45

Amino acids:
Basic, yielded by casein (LEAVENWORTH) 315

Anesthesia:
Ether, acidosis, rôle of lactic acid in (RONZONI, KOECHIG, and EATON) 465

Anthocyan:
Grapes, Clinton (ANDERSON and NABENHAUER) 97
—, Seibel (ANDERSON) 685

Antiketogenesis:
(SHAFFER and FRIEDEMANN) 585

Arginine:
Growth, relation to (ROSE and COX) 747

Atrophy:
Muscle tissue (CHEN, MEEK, and BRADLEY) 807

Autolysis:
Studies (Chen, Meek, and Bradley) 807
— (Hertzman and Bradley) 275

B

Blood:
Acid-base equilibrium (Hawkins) 147
Chlorides of, in pathological conditions (Gram) 337
Cholesterol in (Leiboff) 177
Coagulation, hydrogen ion concentration changes (Hirsch) 795
Dog, chemical changes after thyroparathyroidectomy (Greenwald) 649
Electrolyte changes in ether acidosis (Austin, Cullen, Gram, and Robinson) 829
— equilibrium in (Hastings, Sendroy, Murray, and Heidelberger) 317
Gas determination by manometric extraction (Harington and Van Slyke) 575
—, — — — extraction (Van Slyke and Neill) 523
—, — — vacuum extraction (Harington and Van Slyke) 575
—, — — — extraction (Van Slyke and Neill) 523
Blood—*continued:*
Gas equilibrium in (Hastings, Sendroy, Murray, and Heidelberger) 317
Hydrogen ion, colorimetric determination, at body temperature without buffer standards (Hastings and Sendroy) 695
Parathyroidectomized dog, toxin in (Greenwald) 33
Phenols in (Theis and Benedict) 67
Buffer:
Standards, colorimetric determination of blood pH at body temperature without (Hastings and Sendroy) 695
Butyl alcohol:
Fermentation, acetone-butyl alcohol, *l*-leucic acid formation in (Schmidt, Peterson, and Fred) 163

C

Calcium:
Animal substances (Dienes) 77
Carbohydrate:
Diet, low, low nitrogen metabolism with, in diabetes (Petrén) 355
Metabolism of normal individuals, effect of insulin on (Blatherwick, Bell, and Hill) 241
Carbon:
Combustion method, wet, nitrogen determination in connection with (Anderson and Schutte) 57

Carbon dioxide:
Serum, in presence of ether by Van Slyke method (Austin) 345
Carbon monoxide:
Acidity of hemoglobin, effect on (Hastings, Sendroy, Murray, and Heidelberger) 317
Casein:
Amino acids, basic, yielded by (Leavenworth) 315
Catalysis:
Iron salts, mechanism (Baudisch and Welo) 261
Cerasin:
Lignoceric acid from (Levene, Taylor, and Haller) 160
Chloride:
Blood in pathological conditions (Gram) 337
Corpuscles in pathological conditions (Gram) 337
Serum in pathological conditions (Gram) 337
Chloroacetyl-glycine:
(Levene, Simms, and Pfaltz) 450
Chloroacetyl-sarcosine:
(Levene, Simms, and Pfaltz) 450
Cholesterol:
Blood (Leiboff) 177
Chymase:
Activity (Bodansky) 367
Coagulation:
Blood, hydrogen ion concentration changes (Hirsch) 795
Cod liver oil:
Growth of young chickens, effect on (Dunn) 129
Combustion method:
Carbon, nitrogen determination in connection with (Anderson and Schutte) 57
Corpuscles:
Chlorides of, in pathological conditions (Gram) 337

D

Diabetes:
Carbohydrate diet, low, low nitrogen metabolism with (Petrén) 355
Exercise in, effect (Loebel, Barr, Tolstoi, and Himwich) 9
Phlorhizin, lactic acid formation in (Loebel, Barr, Tolstoi, and Himwich) 9
—, mechanism (Nash and Benedict) 423
Diet:
Carbohydrate, low, low nitrogen metabolism with, in diabetes (Petrén) 355
Digestibility:
Isoelectric point, relation to (Hertzman and Bradley) 275

E

Edestin:
Dakin's method applied to (Osborne, Leavenworth, and Nolan) 309

Electrolyte:
Blood, changes in ether acidosis (Austin, Cullen, Gram, and Robinson) 729
Equilibrium in blood (Hastings, Sendroy, Murray, and Heidelberger) 317
Enzyme:
Milk-coagulating, of *Solanum elaeagnifolium* (Bodansky) 365
Equilibrium:
Electrolyte, in blood (Hastings, Sendroy, Murray, and Heidelberger) 317
Gas, in blood (Hastings, Sendroy, Murray, and Heidelberger) 317
Ether:
Acidosis, blood electrolyte changes in (Austin, Cullen, Gram, and Robinson) 729
Anesthesia, acidosis, rôle of lactic acid in (Ronzoni, Koechig, and Eaton) 465
Carbon dioxide in serum in presence, by Van Slyke method (Austin) 345
Exercise:
Diabetes, effect in (Loebel, Barr, Tolstoi, and Himwich) 9

F

Fat:
Milk rations high in, effectiveness of X substance in preventing sterility in rats on (Mattill, Carman, and Clayton) 729
Fat—*continued:*
-Soluble vitamins (Steenbock and Black) 405
— — (Steenbock, Hart, and Jones) 775
Furfural:
Colorimetric method for estimation (Youngburg and Pucher) 741

G

Gas:
Blood, determination by manometric measurement (Harington and Van Slyke) 575
—, — — — measurement (Van Slyke and Neill) 523
—, — — vacuum extraction (Harington and Van Slyke) 575
—, — — — extraction (Van Slyke and Neill) 523
Equilibrium in blood (Hastings, Sendroy, Murray, and Heidelberger) 317
Glucose:
Alcoholic extract of commercial granular, effect on urinary reducing substance (Wang and Felsher) 659
Glycol aldehyde:
Action (Shaffer and Friedemann) 585
Glycosuria:
Phlorhizin, insulin and (Colwell) 289
Glycuronic acid:
Menthol, in urine (Quick) 667
—, synthesis, in rabbit (Quick) 679

Glycyl-sarcosine:
(Levene, Simms, and Pfaltz) 450
Glyoxal:
Action (Shaffer and Friedemann) 585
Grape:
Clinton, anthocyans in (Anderson and Nabenhauer) 97
Pigments, chemistry (Anderson) 685
—, — (Anderson and Nabenhauer) 97
Seibel, anthocyans in (Anderson) 685
Growth:
Arginine, relation (Rose and Cox) 747
Chickens, young, effect of cod liver oil on (Dunn) 129
Histidine, relation (Rose and Cox) 747
-Promoting properties, induction in ration by exposure to ultra-violet light (Steenbock and Black) 405
— vitamin, yeast, tested for effect on animals (Deas) 5
Scurvy, acute, effect (Anderson and Smith) 181

H

Heat:
Milk, heat treatment of, on mineral metabolism of infants (Daniels and Stearns) 225
Hemoglobin:
Acidity of, effect of carbon monoxide on (Hastings, Sendroy, Murray, and Heidelberger) 317
Histidine:
Growth, relation to (Rose and Cox) 747
Honey:
Invertase in (Nelson and Cohn) 193
Hormone:
Ovarian, extraction and properties (Doisy, Ralls, Allen, and Johnston) 711
Hydrazine sulfate:
Injections in dogs, alkalosis produced by (Hendrix and McAmis) 45
Hydrogen ion:
Blood, colorimetric determination, at body temperature without buffer standards (Hastings and Sendroy) 695
Concentration of blood with coagulation, changes (Hirsch) 795
Hydrolysis:
Methylated peptides, rate (Levene, Simms, and Pfaltz) 445
Peptides, rate, relation of chemical structure to (Levene, Simms, and Pfaltz) 445

I

Insulin:
Carbohydrate metabolism of normal individuals, effect on (Blatherwick, Bell, and Hill) 241
Phlorhizin glycosuria (Colwell) 289
Invertase:
Honey (Nelson and Cohn) 193

Iron:
Salts, catalytic action, mechanism (Baudisch and Welo) 261
Isoelectric point:
Digestibility, relation to (Hertzman and Bradley) 275
Isomerization:
Isostrophanthidin (Jacobs and Collins) 387
α-Isostrophanthic acid:
(Jacobs and Collins) 399
α-Isostrophanthic dimethyl ester benzoate:
(Jacobs and Collins) 400
α-Isostrophanthidic acid:
Oxidation after isomerization with alkali (Jacobs and Collins) 401
α-Isostrophanthidic methyl ester:
(Jacobs and Collins) 395
α-Isostrophanthidic methyl ester benzoate:
(Jacobs and Collins) 396
α-Isostrophanthidic methyl ester oxime:
(Jacobs and Collins) 396
Isostrophanthidin:
Isomerization (Jacobs and Collins) 387
Oxidation with hypobromite (Jacobs and Collins) 393
α-Isostrophanthidin:
Oxidation after isomerization with alkali (Jacobs and Collins) 401
α-Isostrophanthidin oxime:
(Jacobs and Collins) 393

K

Ketolytic reaction:
(Shaffer and Friedemann) 585

L

Lactic acid:
Formation in phlorhizin diabetes (Loebel, Barr, Tolstoi, and Himwich) 9
Rôle in acidosis of ether anesthesia (Ronzoni, Koechig, and Eaton) 465
***l*-Leucic acid:**
Acetone-butyl alcohol fermentation, formation in (Schmidt, Peterson, and Fred) 163
Light:
Ultra-violet, exposure to, induction of calcifying properties in ration by (Steenbock and Black) 405
—, — —, — of growth-promoting properties in ration by (Steenbock and Black) 405
Lignoceric acid:
(Levene, Taylor, and Haller) 157
Liver:
Parathyroid tetany (Blumenstock and Ickstadt) 91

M

Magnesium:
Animal substances (Dienes) 77
Yeast nucleic acid as magnesium compound (Baumann) 1

l-Malic acid:
Product of alcoholic fermentation by yeast (Dakin) 139
Menthol glycuronic acid:
Synthesis, in rabbit (Quick) 679
Urine (Quick) 667

Metabolism:
Carbohydrate, of normal individuals, effect of insulin on (Blatherwick, Bell, and Hill) 241
Mineral, of infants, heat treatment of milk on (Daniels and Stearns) 225
Nitrogen, low, with low carbohydrate diet in diabetes (Petrén) 355
Pentose (Youngburg and Pucher) 741
Phosphorus, of normal individuals, effect of insulin on (Blatherwick, Bell, and Hill) 241
Microchemical gravimetric technique:
(Dienes) 73
Milk:
Breast, dried, feeding (Smith) 625
Enzyme, milk-coagulating, of *Solanum elaeagnifolium* (Bodansky) 365
Heat treatment, on mineral metabolism of infants (Daniels and Stearns) 225
Nutritive properties (Mattill, Carman, and Clayton) 729
Milk—*continued:*
Rations high in fat, sterility in rats on, effectiveness of X substance in preventing (Mattill, Carman, and Clayton) 729
Molybdic method:
Phosphorus, inorganic, in serum (Benedict and Theis) 63
Muscle:
Protein concentration in muscle of calf, cow, and rabbit (Howe) 493
Proteins, soluble, differential extraction (Howe) 493
—, —, precipitation (Howe) 493
Tissue, atrophy (Chen, Meek, and Bradley) 807
Myoalbumin:
(Howe) 515
Myoglobulin:
(Howe) 515
Myosinogen:
(Howe) 515

N

Nitrogen:
Alfalfa plant, basic nitrogen (Vickery) 117
— —, juice, nitrogenous constituents (Vickery) 117
Determination in connection with wet combustion method for carbon (Anderson and Schutte) 57
Metabolism, low, with low carbohydrate diet in diabetes (Petrén) 355

O

Oil:
Cod liver. *See* Cod liver oil.
Peanut. *See* Peanut oil.
Ovary:
Hormone, ovarian, extraction and properties (Doisy, Ralls, Allen, and Johnston) 711

P

Paramyosinogen:
(Howe) 514
Parathyroid:
Tetany, liver in (Blumenstock and Ickstadt) 91
Parathyroidectomy:
Blood of parathyroidectomized dogs, toxin in (Greenwald) 33
Peanut oil:
Lignoceric acid from (Levene, Taylor, and Haller) 158
Pentose:
Metabolism (Youngburg and Pucher) 741

Peptides:
Hydrolysis, rate, relation of chemical structure to (Levene, Simms, and Pfaltz) 445
Methylated, physical constants (Levene, Simms, and Pfaltz) 445
—, rate of hydrolysis (Levene, Simms, and Pfaltz) 445
—, synthesis (Levene, Simms, and Pfaltz) 445
Phenols:
Blood (Theis and Benedict) 67
Phlorhizin:
Diabetes. *See* Diabetes.
Glycosuria. *See* Glycosuria.
Phosphorus:
Animal substances (Dienes) 77
Inorganic, serum, molybdic method for (Benedict and Theis) 63
Metabolism of normal individuals, effect of insulin on (Blatherwick, Bell, and Hill) 241
Pigment:
Grape, chemistry (Anderson) 685
—, — (Anderson and Nabenhauer) 97
Pork:
Production on restricted rations, sunlight in relation to (Steenbock, Hart, and Jones) 775
Precipitation:
Proteins, soluble, of muscle (Howe) 493
Protein:
Concentration in muscle of calf, cow, and rabbit (Howe) 493
Intake, effect of temperature on (Denis and Borgstrom) 109
Muscle, soluble, differential extraction (Howe) 493
—, —, precipitation (Howe) 493
Spinach leaves (Chibnall) 303

S

Saliva:
Organic constituents (Updegraff and Lewis) 633
Salts:
Iron, catalytic action, mechanism (Baudisch and Welo) 261
Sarcosine:
Chloroacetyl (Levene, Simms, and Pfaltz) 450
Glycyl (Levene, Simms, and Pfaltz) 450
Sarcosyl-glycine:
(Levene, Simms, and Pfaltz) 451
Sarcosyl-sarcosine:
(Levene, Simms, and Pfaltz) 451
Scurvy:
Growth of guinea pigs, effect on (Anderson and Smith) 181
Nutrition of guinea pigs, effect on (Anderson and Smith) 181
Serum:
Carbon dioxide in, in presence of ether by Van Slyke method (Austin) 345
Chlorides of, in pathological conditions (Gram) 337
Phosphorus, inorganic, molybdic method (Benedict and Theis) 63
Solanum elaeagnifolium:
Milk-coagulating enzyme (Bodansky) 365
Spinach:
Protein from leaves (Chibnall) 303
Spinacin:
Protein from spinach leaves (Chibnall) 303
Sterility:
Rats on milk ration high in fat, effectiveness of X substance in preventing (Mattill, Carman, and Clayton) 729
Strophanthin:
(Jacobs and Collins) 387
Sunlight:
Pork production, relation to, on restricted rations (Steenbock, Hart, and Jones) 775

T

Temperature:
Protein intake, effect on (Denis and Borgstrom) 109
Tetany:
Parathyroid, liver in (Blumenstock and Ickstadt) 91
Thyroparathyroidectomy:
Chemical changes in blood of dogs after (Greenwald) 649
Tissue:
Muscle, atrophy (Chen, Meek, and Bradley) 807
Toxin:
Blood of parathyroidectomized dogs (Greenwald) 33

U

Urine:
Menthol glycuronic acid in (Quick) 667

V

Valine:
Zein, valine in (DAKIN) 137

Vitamin:
Fat-soluble (STEENBOCK and BLACK) 405
— (STEENBOCK, HART, and JONES) 775
Growth-promoting, yeast, tested for effect on animals (DEAS) 5

Vitamin B:
Adsorption by kaolin (LEVENE and VAN DER HOEVEN) 438
Concentration (LEVENE and VAN DER HOEVEN) 429

Vitamin B—*continued:*
Precipitation with barium hydroxide (LEVENE and VAN DER HOEVEN) 440

Y

Yeast:
Alcohol fermentation by, *l*-malic acid as a product (DAKIN) 139
Growth-promoting vitamin tested for effect on animals (DEAS) 5

Yeast nucleic acid:
Magnesium compound, yeast nucleic acid as (BAUMANN) 1

Z

Zein:
Valine in (DAKIN) 137

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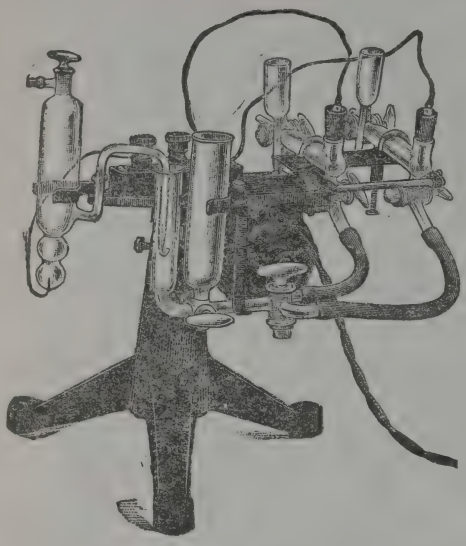

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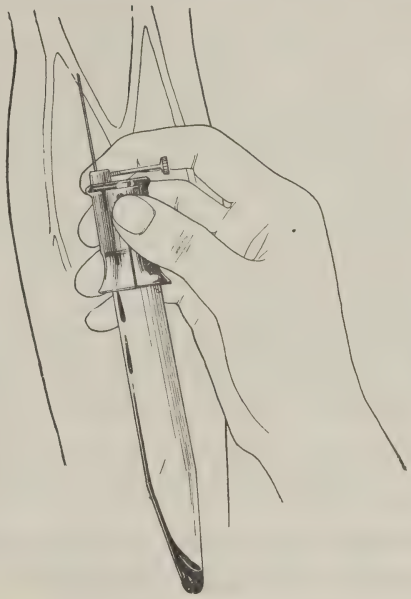

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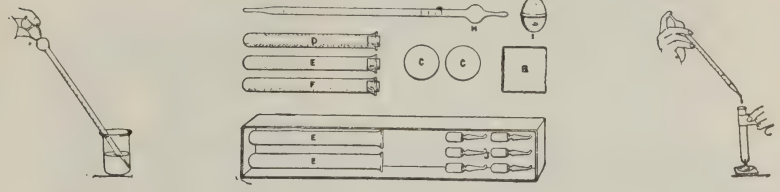

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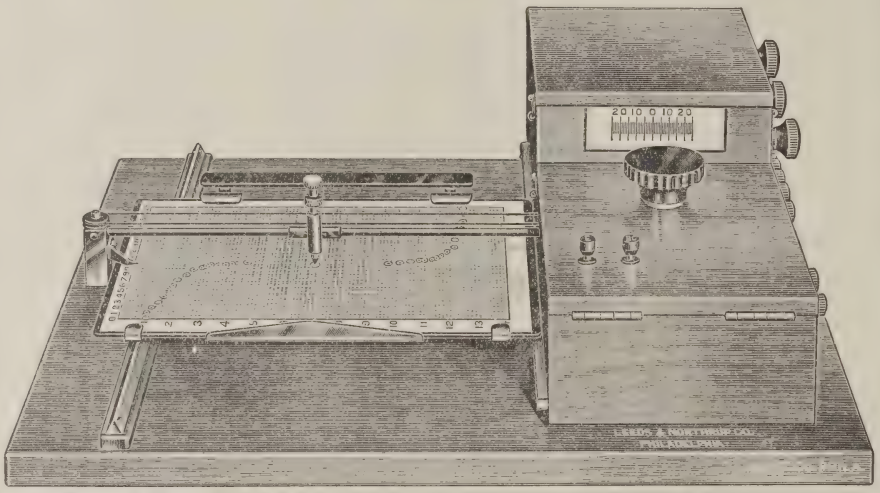

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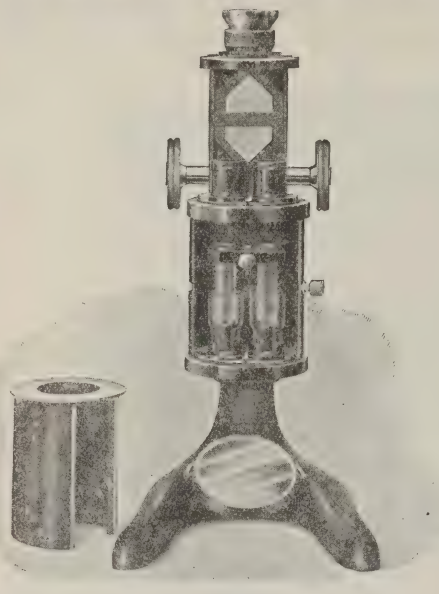

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CONTENTS OF VOL. VII, No. 1. SEPTEMBER 20, 1924

COULTER, CALVIN B. Membrane equilibria and the electric charge of red blood cells.

BODINE, JOSEPH HALL. Some physiological actions of cyanides.

NORTHROP, JOHN H., and KUNITZ, M. The combination of salts and proteins. I.

KLOPP, JOHN W. Observations on the injurious effects of high temperatures on frog skin.

COHN, EDWIN J., and BERGGREN, RUTH E. L. Studies in the physical chemistry of the proteins. III. The relation between the amino acid composition of casein and its capacity to combine with base.

COHN, EDWIN J., BERGGREN, RUTH E. L., and HENDRY, JESSIE L. Studies in the physical chemistry of the proteins. IV. The relation between the composition of zein and its acid and basic properties.

OLIVER, JEAN, and BARNARD, LEONARD. The influence of electrolytes on the stability of red blood corpuscle suspensions.

CROZIER, W. J. On the critical thermal increment for the locomotion of a diplopod.

CROZIER, W. J., and FEDERIGHI, H. Critical thermal increment for the movement of *Oscillatoria*.

CROZIER, W. J., and FEDERIGHI, H. Phototropic circus movements of *Limax* as affected by temperature.

AMBERSON, W. R., MAYERSON, H. S., and SCOTT, W. J. The influence of oxygen tension upon metabolic rate in invertebrates.

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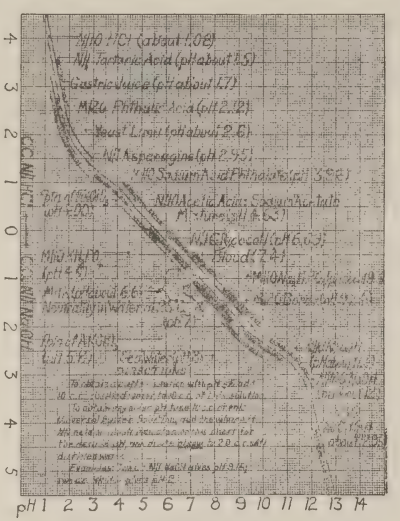

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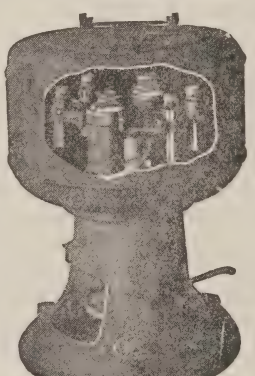

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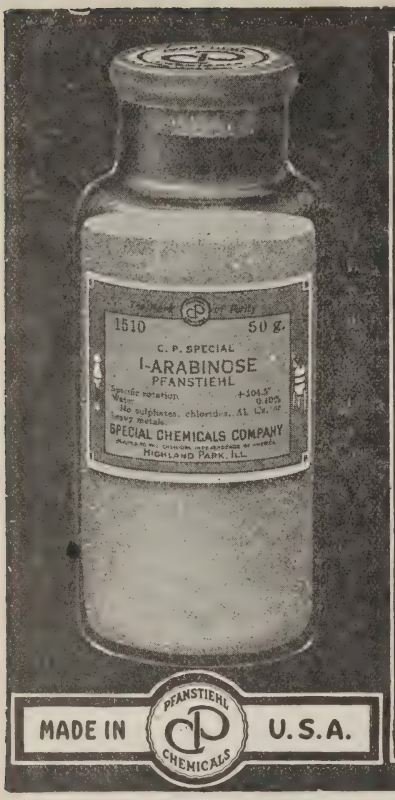

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CONTENTS

SHAFFER, P. A., and FRIEDEMANN, T. E. Antiketogenesis. V. The ketolytic reaction; action of glycol aldehyde and of glyoxal 585

SMITH, LAWRENCE WELD. The experimental feeding of dried breast milk 625

UPDEGRAFF, HELEN, and LEWIS, HOWARD B. A quantitative study of some organic constitutents of the saliva 633

GREENWALD, ISIDOR. Some chemical changes in the blood of dogs after thyroparathyroidectomy 649

WANG, CHI CHE, and FELSHER, AUGUSTA R. The effect of alcoholic extract of commercial granular glucose on urinary reducing substance 659

QUICK, ARMAND J. A method for the quantitative determination of menthol glycuronic acid in urine 667

QUICK, ARMAND J. The synthesis of menthol glycuronic acid in the rabbit 679

ANDERSON, R. J. A contribution to the chemistry of grape pigments. III. Concerning the anthocyans in Seibel grapes 685

HASTINGS, A. BAIRD, and SENDROY, JULIUS, JR. Studies of acidosis. XX. The colorimetric determination of blood pH at body temperature without buffer standards 695

DOISY, EDWARD A., RALLS, J. O., ALLEN, EDGAR, and JOHNSTON, C. G. The extraction and some properties of an ovarian hormone 711

MATTILL, H. A., CARMAN, J. S., and CLAYTON, M. M. The nutritive properties of milk. III. The effectiveness of the X substance in preventing sterility in rats on milk rations high in fat 729

YOUNGBURG, GUY E., and PUCHER, GEORGE W. Studies on pentose metabolism. I. A colorimetric method for the estimation of furfural 741

ROSE, WILLIAM C., and COX, GERALD J. The relation of arginine and histidine to growth 747

STEENBOCK, H., HART, E. B., and JONES, J. H. Fat-soluble vitamins. XVIII. Sunlight in its relation to pork production on certain restricted rations 775

HIRSCH, EDWIN F. Changes in the hydrogen ion concentration of the blood with coagulation 795

CHEN, K. K., MEEK, WALTER, and BRADLEY, H. C. Studies of autolysis. XII. Experimental atrophy of muscle tissue 807

AUSTIN, J. H., CULLEN, G. E., GRAM, H. C., and ROBINSON, H. W. The blood electrolyte changes in ether acidosis 829

Index to Volume LXI 841

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